DEPARTMENT OF BIOMEDICAL ENGINEERING

University of Strathclyde Engineering

An in vitro Feasibility Study of Early Wound Infection Diagnosis and Bacterial Strain Differentiation using pH Change

Master Thesis

By

Laura Maria Bücheler

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This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

Name: Laura Buecheler

Signed:

Date:

Abstract

About 25% of all diabetics have foot ulcers at one point in their life and this is also the leading cause of non-traumatic amputations in developing countries. Bacterial infection of a normally healing wound is suggested to induce delayed wound healing and development to a chronic wound. The pH in chronic wounds is alkaline which inhibits the immune response, reduces the efficiency of antibiotics and antiseptics, and also inhibits tissue enzyme response. This study investigates if there is a link between pH and bacterial colonisation in vitro. If successful, this could enable early bacterial infection detection and therefore improve patient treatment, healing rates and reduce antibiotic misuse. Furthermore, the disposable sensor technology used in this thesis can be integrated into wound dressings and used by itself or in a multi-array-sensor for continuous wound parameter monitoring.

25 hour growth curve experiments were conducted in LB media and FBS for *S. aureus* and *S. epidermidis.* Furthermore a long term growth experiment of both bacteria in FBS was implemented. In all three experiments, a significant 2 pH level drop of the media for *S. aureus* inoculated cultures could be identified while the pH drop for *S. epidermidis* was not that drastic. For both bacteria, however, the pH increased to higher alkaline values when the bacteria settles into stationary growth phase. More research will have to be conducted to verify these results in vivo and with different bacterial strains. It is suggested that *S. aureus* uses mild acid stimulation to alter its gene expression after the pH drop, which in turn changes its metabolism to produce alkaline end products.

Acknowledgments

I wanted to thank Stephen Milne and Andrew Ward for their support at every point of my project. I couldn't have done everything that I did in this short amount of time without your help and excellent guidance.

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1. Introduction

One of the biggest burdens to health care systems all over the world, with an annual expense of US$ 25 billion, are chronic wounds. The three main types of chronic wounds are diabetic foot ulcers, venous and pressure ulcers [Mustoe et al. (2006)]. About 6.5 million patients are currently suffering from a chronic wounds and the number is expected to increase with the rise in obese and diabetic patients and a generally aging population [Chandan et al. (2009), Sen et al. (2009)].

A major challenge in wound care, and one of the causes of chronic wound development, is the rapid identification of infections [Mustoe et al. (2006)]. Unpleasant developed bacterial infections shows symptoms including redness, swelling, pain, spreading erythema, crusting and purulent discharge [NHS (2015), DaCosta et al. (2015) and Keith et al. (2004)]. However, infections are difficult to detect in the early stages. Diabetic foot infections (DFI) for example do not display overt signs including warmth, tenderness and therefore erythema and make it more difficult to detect infections early on [McArdle et al. (2014)].

Microbiological testing will identify the infection bacteria, give useful information about antibiotic susceptibility and semi-quantitative bacterial growth rates. This is done by using selective growth media, staining techniques or other laboratory testing [Stokes et al. (1987), Spicer et al. (2000)]. The biggest drawback of microbiological testing is the duration of the tests, which typically take about 3-5 days and the fact, that only the bacterial load of the wound centre is analysed [DaCosta et al. (2015)]. A new technology is needed to evaluate chronic wound healing continuously and in real time. This will enable clinicians to detect bacterial infection before a severe infection becomes established and biofilms can form.

Furthermore, it would help to reduce antibiotic misuse by ensuring that antibiotics are only prescribed when required.

In this thesis a feasibility study is conducted of whether the presence of bacteria will change the wound pH in a detectable level allowing bacterial differentiation to be carried out as early as possible. If a unique pH profile for bacteria presence in chronic wounds can be identified, early bacteria presence can be detected and the infection treated without any delays .The pH sensor used in this scenario could be integrated in the future to be embedded in bandages and placed directly on the wound. It is also possible to combine different sensors including for example a moisture or temperature sensor to collect other relevant information about the healing process of the wound. Personalised treatment with the help of a miniature, cost efficient, disposable sensor could shorten healing times and lower the healthcare cost burden worldwide.

2. Background

This section reviews the important literature on wound care, the microbiology of infections and wound pH. First of all, the general environment of a chronic wound will be discussed and compared to a normally healing wound. Secondly, the pH environment of wounds, together with current measurement techniques and the effect of bacterial infections with respect to wound healing and its pH are described. Thirdly, the bacteria used in this thesis and relevant microbiological background are presented. Lastly, the choice and working principle of the pH sensor is discussed in further detail.

2.1 Clinical Microbiology of Chronic Wounds

This section describes in more detail the concepts of normal wound healing and how chronic wounds develop and in which way they differ from normally healing wounds. Furthermore the burden of chronic wounds on the society and health care systems is discussed in a bigger scope. Finally the presence and impact of bacteria in chronic wounds is further presented.

2.1.a Normal Wound Healing

The healing of wounds is a complex process involving various reactions and interactions of cells and mediators and is not yet completely understood [Broughton et al. (2006)]. However, there are three main phases of wound healing; inflammation, proliferation and maturation and remodelling [McArdle et al. (2014), Broughton et al. (2006), Wild et al. (2010) and Gosain et al. (2004)]. Figure 1 shows a diagram of the different healing stages including homeostasis which is the immediate response of the wound to bleeding [Gosain et al. (2004)]. Homeostasis is per definition sometimes included in the inflammation stage.

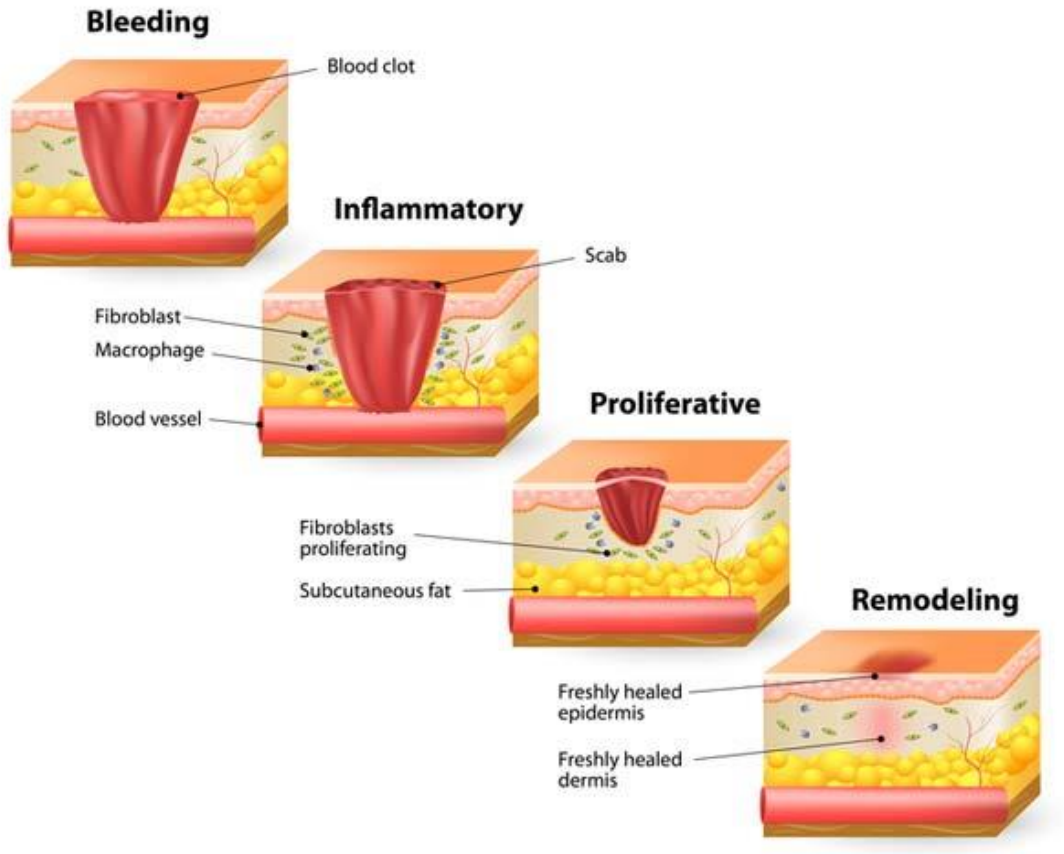


Figure 1: The wound healing stages from bleeding over inflammation, proliferation to maturation and remodelling of the wound [Gosain et al. (2004)].

The inflammation stage lasts about 4-6 days. Initially the Haemostasis and vasoconstriction sets in. Hereby thrombocytes align onto the injured vessel, a coagulation cascade is activated and fibrin is produced to form a hemostatic clot. This prevents further blood loss from the insured tissue. Neutrophils clear the wound of foreign particles and microorganisms. This is followed by the recruitment of Monocytes and the release of growth factors [Broughton et al. (2006), Mahon et al. (2015)].

The proliferation stage starts about 4-5 days after injury and typically lasts a few weeks. This phase is also called the tissue formation phase. Next to new blood vessel formation (i.e.

angiogenesis) a new vascular alignment forms which is clinically known as granulation tissue. This is accompanied by extracellular matrix formation and re-epithelisation starting at the wound edges. In this phase the wound contraction also takes place in which healthy tissue moves centripetally to make the scar area as small as possible [Broughton et al. (2006), Shai et al. (2005)].

The Maturation and remodelling stage can take up to two years after injury. Within the first few weeks Collagen type III is deposited in the wound. During the remodelling phase, equilibrium between degradation of old collagen and the synthesis of Collagen I with a higher stability has to be achieved. This process leads to scar tissue formation. The scar strength will increase from only 5% of the original skin strength to 80% after the end of the maturation and remodelling phase [Broughton et al. (2006), Shai et al. (2005)].

2.1.b Types and Development of Chronic Wounds

A chronic wound is defined as a wound that does not heal within an appropriate time frame, which is mostly defined by clinicians as three to four months [Shai et al. (2005)]. The time is however determined by the size and cause of the wound and the patient's health status. Chronic skin wounds are also called chronic cutaneous ulcer in dermatology [Shai et al. (2005)].

Chronic ulcer development is a complex process and can also be formed by several underlying conditions and mechanisms. The most common ulcers are venous ulcers, ulcers due to peripheral arterial disease, diabetic ulcers, Livedoid vasculitis, pressure ulcers and ulcers developing in the course of cellulitis. Other causes can be due to mechanical trauma, drug side effects, nutritional disorders, metabolic disorders including diabetes mellitus,

neuropathic ulcers, tumoral diseases, hematologic abnormalities, hypercoagulable states, Dysproteinemias, connective tissue disease, Vasculitis or Vascular abnormalities [Mustoe et al. (2006), Shai et al. (2005)].

A chronic wound does not follow normal healing times and remains in a constant inflammation phase [Shai et al. (2005)]. Mustoe et al. (2006) states that abnormalities in chronic wound pathogenesis are caused by a combination of local tissue hypoxia, bacterial colonization of the wound, repetitive ischemia reperfusion injury, and an altered cellular and systemic stress response in the aged patient. More factors of possible chronic wound development are shown in figure 2.

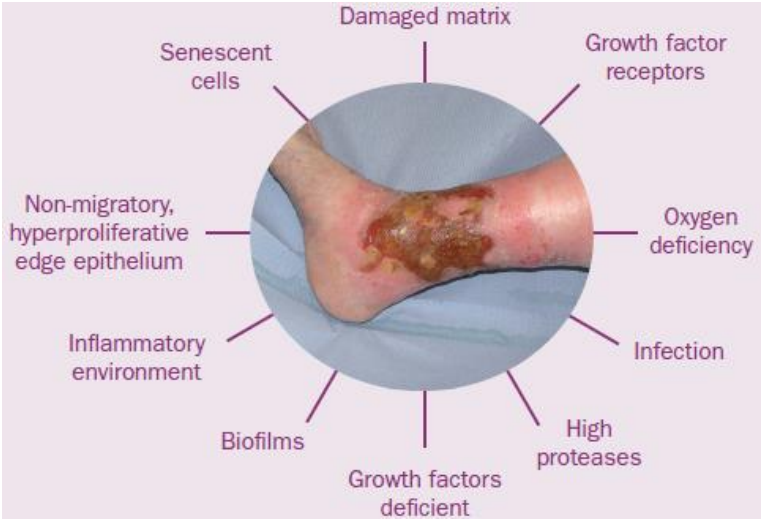


Figure 2: Possible causes for chronic wound development. Presented are possible pathologic abnormalities related to chronic wound development and non-healing of wounds [Drew et al. (2007)].

A number of factors contribute to delayed wound healing in chronic ulcers [Shai et al. (2005), Schneider et al. (2007) and Muller et al. (2007)]. A chronic ulcer can be identified by an increased enzymatic activity of matrix metalloproteases (MMP), reduced response of growth

factors and cell senescence. MMP degrade growth factors and newly formed extracellular matrix components such as collagen and fibronectin. Moreover, the activity of MMP inhibitors is reduced in chronic wounds. Additionally to the degradation of growth factors by MMP's, it is implied that chronic ulcers also show reduced expression of growth factors. Finally, cell senescence (i.e. growing old of cells) leads to reduced proliferation capacity and decreased response to growth factors [Shai et al. (2005), Schneider et al. (2007)].

An ulcer's appearance can be used to diagnose its cause and therefore to determine the best treatment. Features used for ulcer classification are the surface area, depth of the ulcer, its peripheral discoloration and undermining (i.e. its spread and involvement or destruction of peripheral tissue) [Shai et al. (2005)].

2.1.c Bacteria in Chronic Wounds

Historically, a set of objective and subjective criteria were produced to help clinicians determine whether a wound is infected or not. They rely on the individual's sense of smell, touch and the ability to identify different colouring of the wound. Identification of infection differs between wound types, which makes it even harder for clinicians to detect bacterial infections by subjective criteria [Keith et al. (2004), Puchberger-Enengl et al. (2012)]. These methods are out of date and are mostly only used as a control to the swabs or other microbial tests [Puchberger-Enengl et al. (2012].

Bacteria are present at the surface and deep tissue of all wounds. It has not yet been determined if bacteria colonization itself is a delaying wound healing factor [Schneider et al. (2007)]. However, the number of bacteria present in the wound determines if it's a bacterially

infected wound with delayed healing or if only a small amount of bacteria are present and wound healing is even accelerated [Jones et al. 2004].

The species of bacteria and the location where the bacteria are found are important factors in disease pathology. The human body is naturally colonised with different bacterial strains, the so-called “normal flora”. These live in symbioses with the human host and support it by the digestion of food, as a bacterial barrier to prevent pathogenic microbes or the production of vitamins [Murray et al. (2009)]. In contrast, virulent bacteria grow at the expense of the host and opportunistic bacteria take advantage of pre-existing conditions, for example immunosuppression of the patient [Murray et al. (2009)].

An analysis of bacteria colonization on intact skin showed that *S. epidermidis* is the most common bacteria and was present in 80.65% of the time in all subjects at every time point [Wysocki et al. (2012)]. Between two and 16 different bacteria species could be identified on intact skin from all tested patients, with an average of eight per wound. *S. aureus* has been detected in 25.8% of all cases [Wysocki et al. (2012)].

In comparison to the bacteria colonization of intact skin, the bacteria presence of chronic wounds was also assessed by Wysocki et al. (2012). They found that *P. aeruginosa* was the predominant species in the total number of chronic wounds tested with 48.4%. *S. epidermidis* was found in 21% of all cases and in 37.5% of all measurements, *S. aureus* and *S. epidermidis* were found in the intact skin and the wound. A limitation of this study is that the bacteria distribution in a chronic wound is highly heterogeneous [Thomsen et al. (2010)]. A swab taken from deeper tissue, for example, is more likely to contain *P. aeruginosa* while the surface layers are more likely to contain *S. aureus* [Fazli et al. (2009)].

It should be noted that bacteria presence in a chronic wound does not necessarily lead to a bacterial infection with the present microorganisms. The quantity of the bacteria present should therefore always be assessed and the infection treated for the major pathogenic bacteria rather than the fastest growing one [Burmølle et al. (2010)].

S. aureus and *S. epidermidis* are both opportunistic bacteria that are present in the normal flora of the skin. Once the epidermis is broken, they can easily enter the vulnerable tissue beneath it and cause an infection. This is a common problem in patients with indwelling catheters, intravenous lines or chronic wounds [Murray et al. (2009), Wysocki et al. (2012)].

In a study from Hansson et al. (1995) it was found that bacterial numbers in chronic wounds, including *S. aureus* and *S. epidermidis,* remain constant. It was assumed that bacterial presence in chronic wounds is due to a natural flora within the wound bed. This flora appears to restrict overgrowth of one bacterial species which causes an infection of the wound [Hansson et al. (1995)]. Wysocki et al. (2012) confirmed these results by identifying the bacterial strains present in chronic venous ulcers over time. In contrast, Wolcott et al. (2009) identified 13 Gram-positive and Gram negative bacteria, including *S. aureus* and *S. epidermidis,* in chronic wounds that are able to degrade extra cellular matrix proteins which might also contribute to a delayed wound healing . Halbert et al. (1992) showed that *S. aureus* does indeed delay wound healing due to several unfavourable processes.

In chronic wounds the risk of infection is greater. Diabetic patients, for example, have an increased risk of bacterial infection due to the immunosuppressive effect of diabetes and the hypoxic and necrotic nature of wounds [Jones et al (2004)].

At least 60% of all chronic wounds contain a biofilm. These consist of complex bacterial structures and secrete extracellular polymeric substances which act as a biochemical barrier. In the biofilm the bacteria are mostly protected against mechanical forces, shear forces, the host's immune response and antibiotics. This makes it very difficult to treat chronic wounds with biofilms. The knowledge of biofilm formation and treatment is therefore important and should be taken into account when treating chronic wounds [Donelli (2014)].

2.1.d Current Bacteria Detection Techniques

The types of tests conducted will determine the possible findings in the sample and the current techniques are unlikely to detect all bacteria. Especially difficult to detect are low concentrations and slowly growing microorganisms. However, the choice of test is not the only factor influencing the diagnostic results. Open wounds often are contaminated with pathogenic microbes unrelated to the infection itself. A swab should be taken from deeper wound layers after cleaning the wound [Murray et al. (2009), Burmølle et al (2010), Fazli et al (2009) and Mahon et al (2015)].

The best microbiological test for wound sampling is a deep tissue biopsy [Keith et al. (2004), Murray et al. (2009)]. However, it is an invasive, expensive technique that causes pain to the patient. A different approach is the semi-qualitative surface swabs. It is less expensive and causes less pain then the deep tissue biopsy, but no conclusive guideline exists for the most effective swabbing technique [Keith et al. (2004), Murray et al. (2009) and Ono et al. (2015)].

The Levine swab is the most commonly used technique but is limited to analyse only the centre of the wound which has the potential to misidentify an important bacterium at the edges of the wound. Microbiological tests take up to five days to identify a bacterium, but

are much more accurate than the subjective analysis of signs and symptoms mentioned above [DaCosta et al. (2015), Ono et al. (2015)].

In figure 3 an explanatory bacteria identification algorithm is illustrated for different Staphylococci strain identification. This graph only includes a few bacteria and tests, but it can already be seen that there are a wide variety of tests available for microbial laboratory tests [Mahon et al. (2015)].

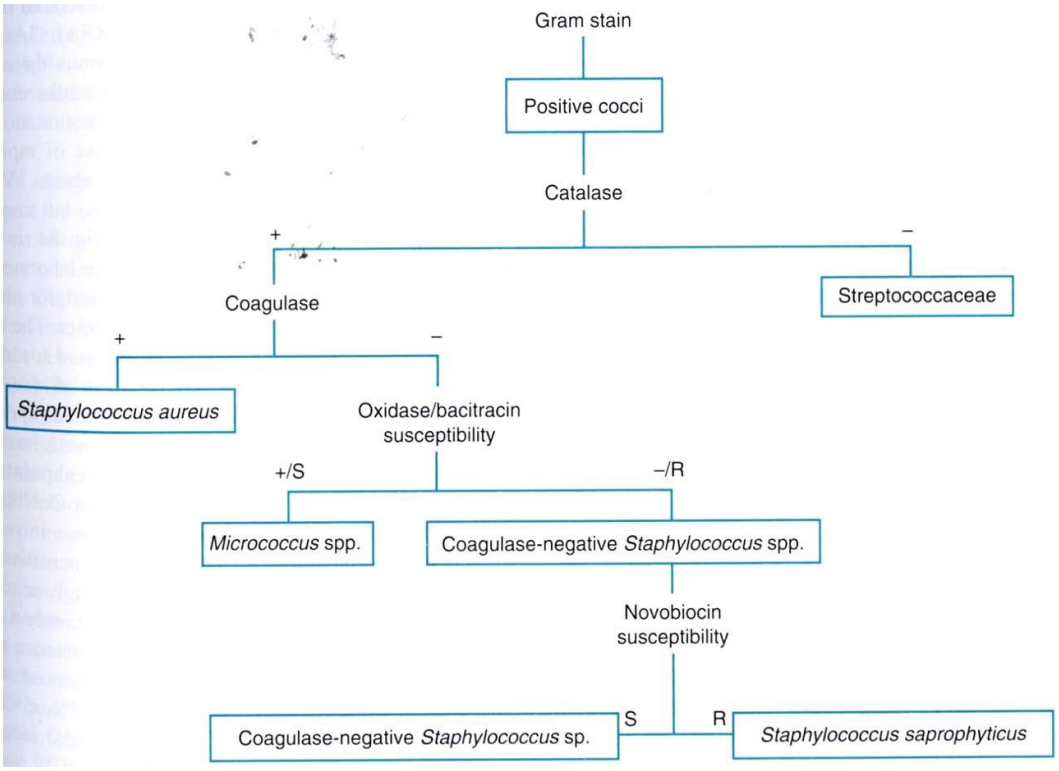


Figure 3: Bacteria identification algorithm for staphylococcal species. It should be noted that this this is not complete and that there are other coagulase-positive bacteria besides S. aureus. S stands for Sensitive and R for Resistant in this case [Mahon et al. (2015)].

2.1.e The Burden of Chronic Wounds

In the United States, chronic wounds affect around 6.5 million patients. It is claimed that an excess of US$25 billion is spent annually on treatment of chronic wounds and the burden is

growing rapidly due to increasing health care costs, an aging population and a sharp rise in the incidence of diabetes and obesity worldwide [Chandan et al. (2012), Sen et al. (2009)]. Chronic wounds are defined as wounds that will not heal for several months or even years. A study in 2007 showed that 24% of all chronic wounds investigated took 6 months or more to heal with 16% having a chronic wound for more than a year. In the same study 12.6% of all chronic wounds were infected [Drew et al. (2007)]. More than 90% of all chronic wounds can be divided into three types of wounds; diabetic ulcers, venous ulcers and pressure ulcers [Mustoe et al. (2006)]. The International Diabetes Federation states that 382 million people were living with diabetes worldwide in 2013 and the probability of these patients to suffer from foot ulcer at one point in their live is 10-25% [Michael et al. (2015), International Diabetes Federation (2014)]. It is furthermore, the leading non-traumatic cause of lower extremity amputation in developing countries and the United States [McArdle et al. (2014)]. Diabetic ulcers alone are a big burden to the health care system which is only further stressed by the additional annual cost of €6.5 billion to treat venous leg ulcers and between €2.2-3.2 billion to treat and prevent pressure ulceration in the UK [Drew et al. (2007)].

Another major burden on society rather than the healthcare systems is antibiotic misuse. Patients with chronic wounds might get prescribed antibiotics to prevent severe infection symptoms developing before the test results are returned. One result of this is the evolution of antibiotic resistance bacteria. Professor Dame Sally Davies claimed in 2013 that antibiotic resistance is “as big a risk as terrorism” [Bazian (2015)]. What we now define as a minor infection could easily develop in a serious threat if the bacteria causing the infection are resistant to all currently known antibiotics. In order to limit the ongoing development of

antibiotic resistant bacteria the antibiotic stewardship was introduced in 2001. It is a sustainable approach of medical institutions to ensure the quality of antibiotics in terms of type, dose, duration and application [|A|B|S| Initiative Deutschland (2011)].

In this study, pH change in simulated wound fluid has been investigated during bacterial growth of two Staphylococcus species with a screen printed pH sensor. If a trend in pH change exists, continuous online monitoring of chronic wounds is possible with the help of an integrated wearable pH sensor in the wound dressings. This way bacterial infections can be detected and treated as early as possible to minimise healing times of chronic wounds and therefore lower the burden on the healthcare systems worldwide. Antibiotic misuse can also be avoided which will further reduce the threat of antibiotic resistance in bacteria on the society.

2.2 Microbiology Theory

In this section the important background about bacteria is explained. This general knowledge is necessary to understand the concepts investigated in the discussion of the thesis.

2.2.a Bacterial Growth

If sufficient nutrients and metabolites are present in the growth media, bacteria can be divide into two identical cells through a process called binary fission [Murray et al. (2009), Mahon et al. (2015)]. The bacterial growth is influenced by several environmental factors including temperature, pH and the gaseous composition of the atmosphere. The metabolic pathways change according to oxygen presence and vary in energy efficiency and metabolic end products [Michael et al. (2002), Mahon et al. (2015)].

Typical bacterial growth consists of four stages (figure 4). A lag phase can be observed in which the freshly inoculated bacteria need time to adapted and grow in the new media and will not yet divide. The next phase is called the log or exponential phase in which the bacterial cell density increases exponentially. After most of the nutrients are metabolised further growth is limited, the growth rate declines and stays reasonably stable in the stationary phase. With increasing nutrients consumption, cell growth or division rates decrease and more toxic metabolites build up in the media. Consequently, the viability of bacteria in the media decreases in the death phase [Murray et al. (2009), Mahon et al. (2015)].

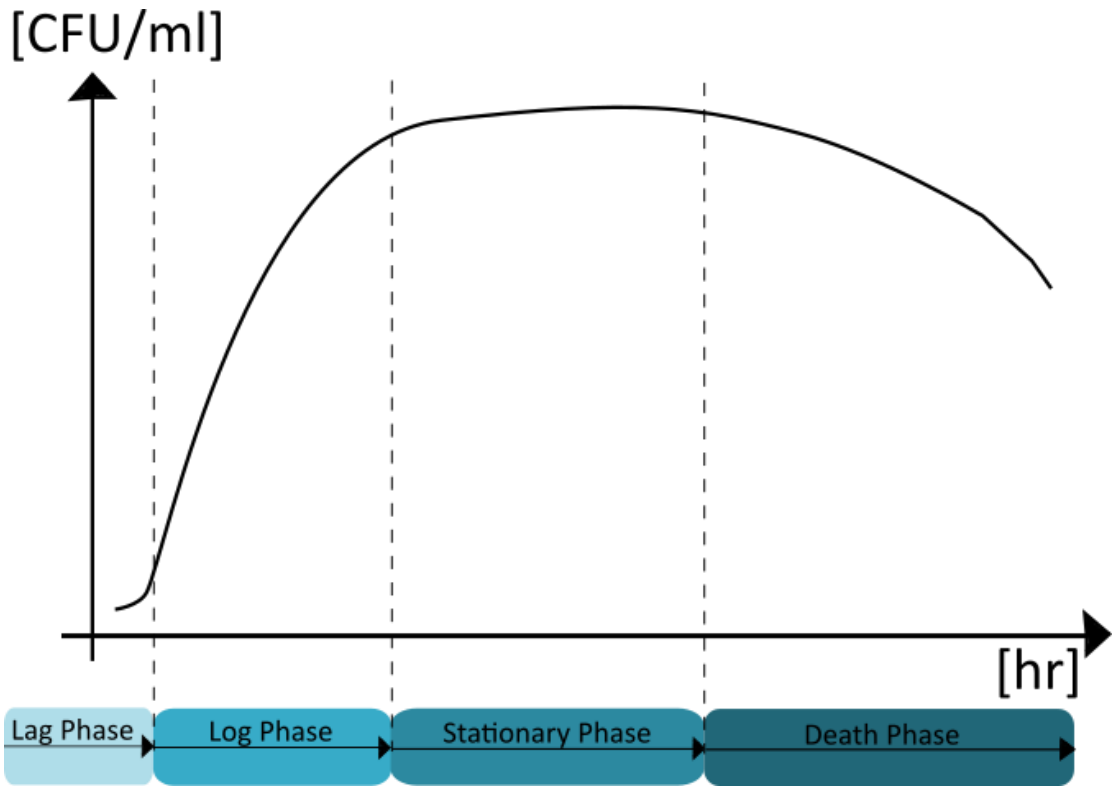


Figure 4: General bacterial growth profile over time. The bacterial cell density stays relatively constant in the lag phase, increases during log phase and at a high constant level in stationary phase. In death phase the number of viable bacteria declines.

2.2.b Gram staining

Originally developed to differentiate between different bacteria strains, Gram straining evolved and is now the best known and most widely used strain for bacteria classification. It divides all bacteria in Gram-positive and Gram-negative bacteria depending on their phenotypic characteristics [Murray et al. (2009)].

The difference between Gram-positive and Gram-negative bacteria lies in their cell wall as illustrated in figure 5. While Gram-positive bacteria have a thick peptidoglycan layer with different included molecules that allows them to survive in media with high salt concentrations, Gram-negative bacteria have a thinner inner layer of peptidoglycan surrounded by an outer membrane of lipopolysaccharides, phospholipids and proteins. This layer makes it hydrophobic which enables Gram-negative bacteria to be antibiotic resistant in some extent [Stokes et al. (1987), Michael et al. (2002), Spicer et al. (2000), Elliott et al. (2011) and Murray et al. (2009)].

Gram-negative bacteria are more resistant to lysozyme, acid and bile while Gram-positive bacteria, for example *S. aureus* and *S. epidermidis*, are fairly resistant to high salt concentrations [Murray et al. (2009)].

Gram staining is the most important test for bacteria characterization because it is a very quick and reliable test. It helps to determine which identification test would be useful to continue with. During the test bacteria are fixed with iodine and washed with alcohol which will stain Gram-positive bacteria the purple of crystal violet while Gram-negative bacteria lose their colour and will be counterstained with a pink dye [Stokes et al. (1987), Michael et al. (2002), Spicer et al. (2000) and Elliott et al. (2011)].

With the help of other nucleic acid-based tests, direct examination, antigen detection, selective cultures and antibody detection methods it can be determined what type of bacteria is present in a sample solution [Murray et al. (2009), Namvar et al. (2014)].

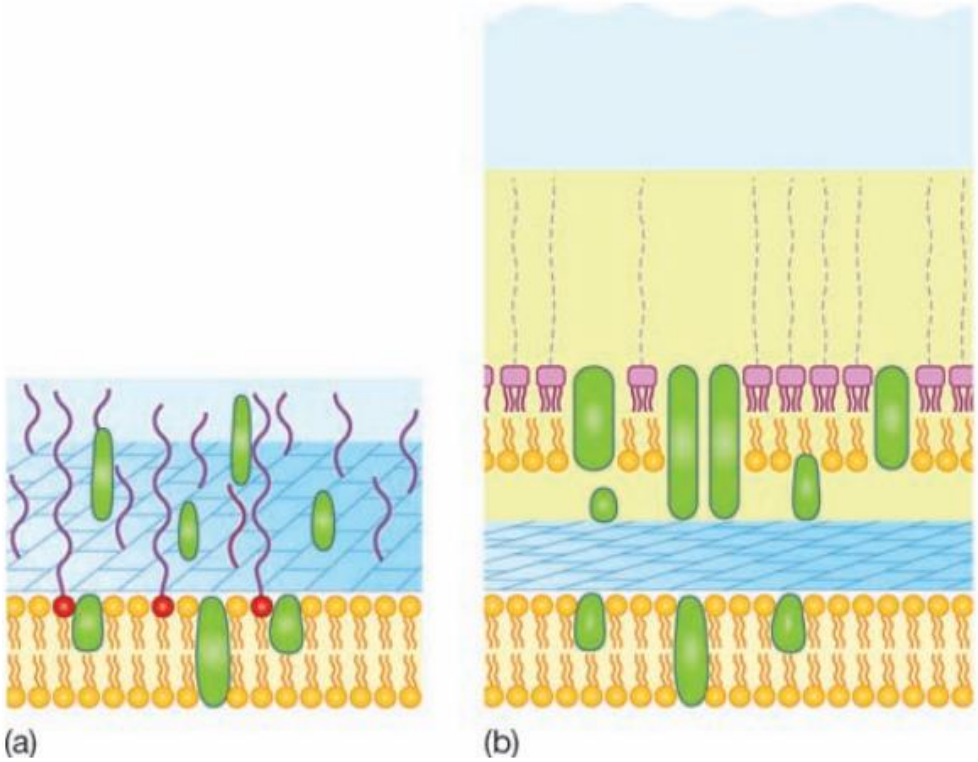


Figure 5: Illustration of the difference of cell walls of (a) gram positive and (b) gram negative bacteria [Elliott et al. (2011)].

2.2.d *S. aureus* and *S. epidermidis*

The two Gram-positive bacteria used in this study are presented in this section and their pathogenic capabilities discussed.

Both bacteria were steaked out on LB agar plates and incubated overnight. The resultant agar plates are shown in figure 6. It can be seen that *S. aureus* forms bigger colonies with a yellow colour, while *S. epidermidis* grows smaller colonies with a white colouring.

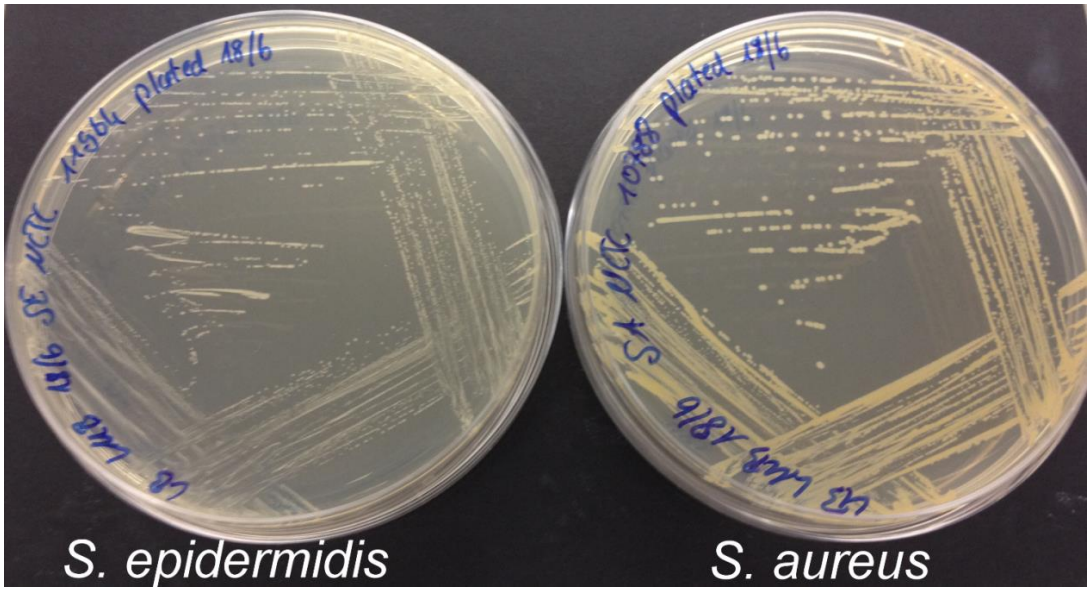


Figure 6: Overnight incubation of S. aureus and S. epidermidis on LB plates. Agar plates of S. epidermidis on the left and S. aureus on the right S. aureus forms bigger colonies and with a deeper yellow colour compared to S. epidermidis.

Staphylococci have a spherical shape, are catalase positive and do not form endospores [Murray et al. (2009)]. *S. epidermidis* used in this study are coagulase negative and commonly found on the surface of the skin as part of the natural flora. They have an approximate cell diameter of 0.5 to 1.5 µm. They grow in grape like clusters [Spicer et al. (2000), Murray et al. (2009), Namvar et al. (2014) and Wysocki et al. (2012)]. The fact that *S. aureus* is commonly found in the normal flora of the skin is one reason for the many hospital-acquired infections with *S. aureus*. They can be transferred through direct contact or via bed linens or other fomites. *S. aureus* can cause a disease through toxin production or the direct invasion and destruction of host tissue [Murray et al. (2009), Wysocki et al. (2012)].

S. epidermidis is a non-pigmented, coagulase-negative, non-spore forming and non-motile bacteria. It is typically not associated with chronic skin ulcer infection [Murray et al. (2009)] but with catheter and shunt infections, endocarditis of artificial valves or prosthetic joint

infections [Murray et al. (2009)]. Especially its ability to attach to artificial material and biofilm production capability increased the number of *S. epidermidis* caused infections in the past decades. It is often antibiotic resistant and can confer its resistance to *S. aureus* by transduction [Spicer et al. (2000), Namvar et al. (2014) and Mahon et al. (2015)].

S. aureus (figure 7) can be coagulase-negative or positive and is associated with various pathological conditions including meningitis and arthritis as well as boils. *S. aureus* is also the cause of the so called scaled skin syndrome, toxic shock syndrome, toxic epidermal necrolysis and food poising [Murray et al. (2009)]. It can be recovered from almost any clinical device and the increased drug resistance is a growing trend [Michael et al. (2002), Kang et al. (2011)]. According to Mahon et al. (2015) *S. aureus* is the predominant bacteria causing skin infections in America with 44.6% of the overall number of isolates [Stokes et al. (1987), Michael et al. (2002), Spicer et al. (2000), Namvar et al. (2014) and Mahon et al. (2015)]. *S. aureus* and *S. epidermidis* are facultative anaerobes. This means they are able to grow with and without oxygen presence [Murray et al. (2009)].

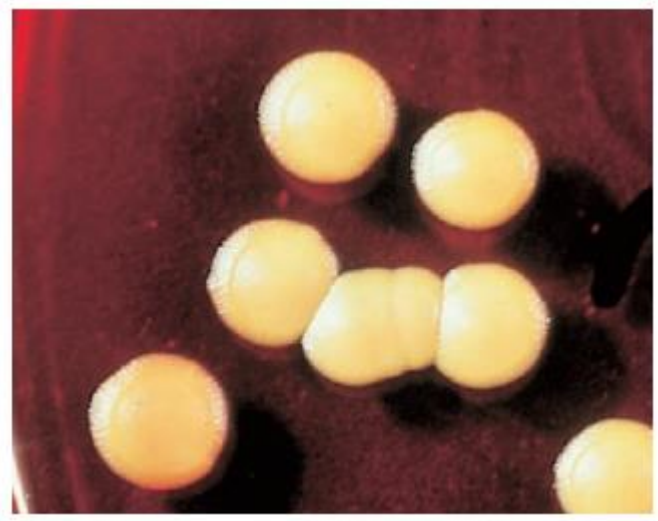


Figure 7: Shows a blood agar plate with S. aureus colonies of a diameter of 2-3mm [Elliott et al. (2011)].

2.2.e Biofilms

A biofilm is a collection of bacteria that are connected in an extracellular matrix with special organization made by the bacteria themselves out of polysaccharides. Bacteria in biofilms are protected by an extracellular matrix and, therefore, protected against major threats including antibiotics. After biofilm formation, bacteria can resist host defences and antibiotics in their gelatinous mass. This makes it difficult to remove all bacteria from the wound using antibiotics. The antibiotics will only attack the bacteria at the surface of the biofilm and cannot migrate into it to kill the bacteria residing within the biofilm. Large pieces of the biofilm can, furthermore, detach from the original biofilm location and spread to other locations. It is also possible for these pieces to be large enough to cause fatal thromboembolism (i.e. clots) [Strelkauskas et al. (2010)].

The most common method to eliminate the biofilm is to surgically remove the biofilm from the wound site [Murray et al. (2009), Burmølle et al. (2010)]. If a biofilm is formed at the

surface of an artificial joint replacement, the only sufficient solution is to remove the joint. Afterwards it can be replaced it with another one a few weeks later after a biofilm in an open wound can also be treated using immune modulation and antimicrobial agents [Mahon et al. (2015)].

The importance of biofilms and the additional risk that comes with it was already mentioned in section 2.1.c Bacteria in Chronic Wounds. Even though *S. epidermidis* is not known to commonly produce biofilm in chronic wounds, it is a major problem in artificial joint replacements and catheters crossing the skin. It is, however, possible for a chronic wound to exhibit a biofilm produced from *S. epidermidis* [Foulston et al. (2014), Donelli (2014) and Mahon et al. (2015)].

2.2.f Metabolism

The metabolism of microorganism describes the biochemical reactions used to break down organic compounds to gain energy and construct new bacterial parts. This is the only process for bacteria to gain energy, in form of ATP, since bacteria do not have mitochondria. Dependent on environmental factors, different metabolic pathways are used to break down substances. This is mostly influenced by regulating specific enzyme productions or enzyme activity. Enzymes, like other proteins, are influenced by pH and working temperature and can change their tertiary structure which in turn inhibits enzyme activity by physically changing the active binding site of the enzyme. Enzyme activity can also be inhibited by removing necessary cofactors to catalyse reactions [Mahon et al. (2015)].

Bacteria can produce energy by two major mechanisms; fermentation and respiration. Fermentation and respiration can be carried out by aerobes and facultative anaerobes [Michael et al. (2002), Mahon et al. (2015) and Strelkauskas et al. (2010)].

Respiration transfers electrons to external electron acceptors including oxygen, nitrate, ferric ion, sulfate or carbonate. It is a more efficient way to produce energy than fermentation. This process uses the Krebs cycle and the electron transport chain to produce energy and one glucose molecule yields a total of 38 ATP. In respiration, oxidative phosphorylation is used to form the high energy compound ATP from ADP. In oxidative phosphorylation some of the energy stored in the cytoplasmic membrane by the proton motive force is released and used to produce ATP [Michael et al. (2002), Mahon et al. (2015) and Strelkauskas et al. (2010)]. The development of the proton motive force is explained in more detail below.

Electron transport carriers are spread in the membrane in a way that protons are separated from electrons as the electrons are transported through the electron transport chain. In the end, an electron is transferred to an external electron acceptor, for example oxygen, while several protons are released into the environment. This leads to a small acidification of the external environment. Furthermore, a pH gradient and an electrochemical potential is produced since the membrane is impermeable for H^+ and OH^-, and H^+ are accumulated in the external media. This potential is about 200 mV and is called the proton motive force and is continuously feeding the membrane with energy. Part of this energy is stored in ATP as described above [Michael et al. (2002), Booth (1985)].

S. epidermidis uses carbohydrates like fructose and maltose for aerobic respiration while it can use glucose for anaerobic conditions. It is, in comparison to *S. aureus*, not able to ferment

mannitol which is a unique diagnostic way to differentiate between the two bacteria [Namvar et al. (2014)]. During fermentation different end products are produced that will alter the pH in a different manner than other by-products. This is the basis of the hypothesis discussed in this study. The pH is used to determine if bacteria are present in the wound and according to the specific pH profiles, the bacterial species might be determined from the change in pH in the wound fluid.

During fermentation an organic compound accepts an electron in an anaerobic environment with the absence of usable external electron acceptors. Fermentation is less efficient than respiration because the initial substance is not completely reduced before the reaction. The fermentation of glucose, called glycolysis, only yields 2 ATP. A mixture of end products is produced including lactate, ethanol, acetoin and butyrate which can be used to determine the bacterial species [Michael et al. (2002), Mahon et al. (2015)]. Common fermentation pathways are illustrated in figure 8 [Strelkauskas et al. (2010)]. It should be noted that fermentation does not rely on the proton motion force to produce energy in the form of ATP [Michael et al. (2002)].

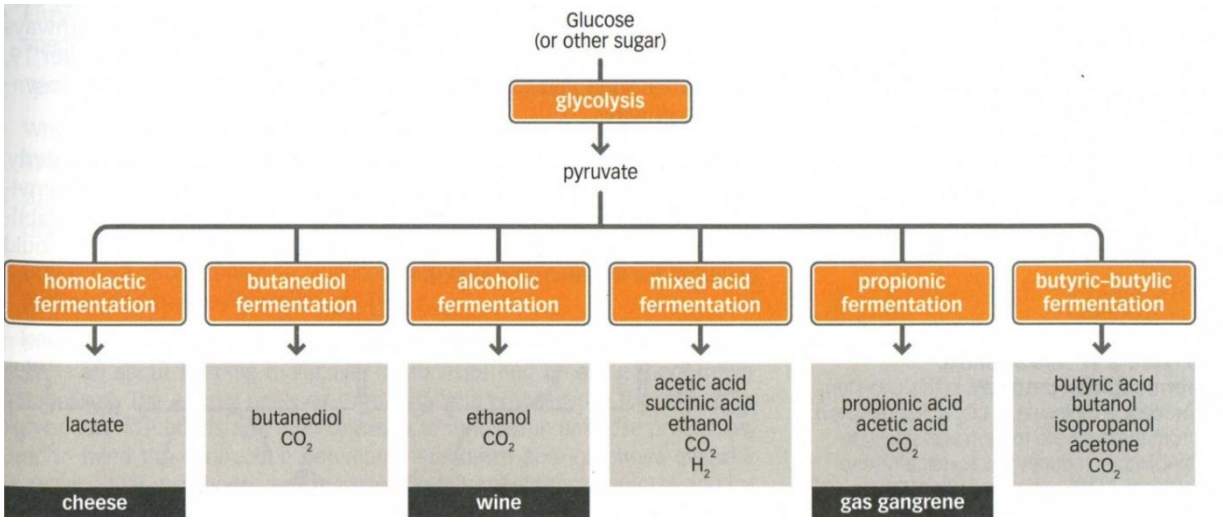


Figure 8: Different fermentation pathways for pyruvate after glycolysis [Strelkauskas et al. (2010)].

Many bacteria produce by-products that can damage the human host for example through acids, gasses or toxins [Murray et al. (2009)]. The human host is trying to fight the infection by activating innate- and immune-responses. These, however, can have damaging consequences to the infected area. The damage caused by both, the bacteria and the consequences of the host response, produce a disease [Murray et al. (2009)].

In aerobic conditions, *S. aureus* mainly produces carbon dioxide and acetic acid during carbohydrate oxidation [Strasters et al. (1963)]. In an anaerobic condition however, the end products are lactic acid and low levels of CO_2 [Strasters et al. (1963)].

Exogenous glucose and free amino acids can be used by *S. aureus* for endogenous respiration [Ramsel et al. (1961)]. One end product of endogenous respiration is ammonia which will be excreted from the bacteria in the environment and therefore, increases the pH level [Ramsel et al. (1961)].

S. aureus and other bacteria can adapted to change in environment with different gene expression which enables them to grow in environments with a pH range of 5-9 [Rode et al. (2010), Weinrick et al. (2004)]. A drastic pH change to acidic levels will lead to an acidic stress situation of the bacteria in which the acid tolerance response (ATR) is activated [Rode et al. (2010)].

In an acidic stress situation the membrane composition changes, the proton efflux increases together with the amino acid catabolism, DNA repair enzymes are activated and proteins that promote survival at low pH values are synthesised [Rode et al. (2010)]. The cytoplasmic pH is strongly regulated using different pathways to ensure no DNA damage that would arise from a low internal pH. The buffering capacity of *S. aureus* is 160-360 nmol H^+/pH per mg protein. Next to a buffering system, the use of active transport systems via an increased synthesis of ATPase to increase proton efflux is used to regulate internal pH. Furthermore, the production of acids or bases can be used to regulate the pH. For example, some Gram-positive bacteria use arginine deiminase which is broken down into ornithine, carbon dioxide and ammonia which will also alkalinize the cytoplasm [Rode et al. (2010), Booth (1985)].

Several studies talk about the correlation of medium pH and metabolic properties of a bacterium. It can be noted that fermentation of acidic end products occurs when the bacteria grow in alkaline pH media and neutral end products are produced when the external pH is acidic. A growing cell typically has an alkaline pH which drops during growth through accumulation of acids in the cytoplasm [Booth (1985)].

The adaption of the gene expression to a lower pH change into slightly acidic values of approximately 5.5 are called Mild Acid Stimulation (MAS) [Weinrick et al. (2004)]. Hereby,

the bacterial metabolism is adapted to the new environment without activating the entire ATR. It is suggested that pH is a factor which is determining the activation and deactivation of different genes and this will have to be further investigated to identify the exact pathways [Weinrick et al. (2004)].

2.3 pH and chronic wounds

In order to put the pH level into context of chronic wounds, the pH level and profile of a chronic wound has been investigated in the literature. In the following subsection the meaning of pH is explained together with known pH changes in chronic and infected wounds.

2.3.a pH Fundamentals

During a pH measurement, the amount of free hydrogen ions (H^+) in a solution is measured. The pH is the negative logarithm to base 10 of the H^+ concentration in mol/l as seen in the equation below [Murray et al. (2009), Mahon et al. (2015), Strelkauskas et al. (2010) and Schneider et al. (2007)].

$$pH = - \log(H^+)$$

Figure 9 shows a pH scale with commonly known substances [Strelkauskas et al. (2010)].

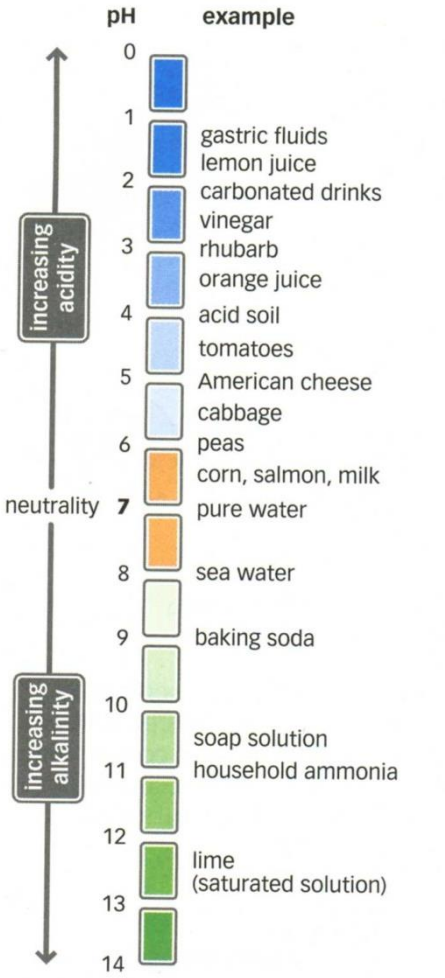


Figure 9: The pH scale with common substances to clarify the concept of alkalinity and acidity [50].

The pH level can be influenced by compounds in the solution itself. Water will dissociate and one water molecule will produce one proton and one hydroxyl ion. This reaction happens continuously and spontaneously in every aqueous solution. Acids and alkalis can also dissociate and produce protons and hydroxyl ions accordingly. A change in temperature will change the measurable pH and big temperature changes should always be documented when doing experimental work [Strelkauskas et al. (2010), Schneider et al. (2007)].

The typical pH scale includes values between 0-14 which corresponds to a proton concentration of $10^0 – 10^{-14}$ mol/l in the sample solution. A pH lover than 7 is per definition acidic, a pH of 7 is neural and all pH values higher than 7 are alkaline [Mahon et al. (2015), Strelkauskas et al. (2010) and Schneider et al. (2007)].

2.3.b pH of Intact Skin

The skin is the biggest body barrier and protects it against bacterial or fungi infections. It is often referred to the acid mantle due to its low pH that ranges from pH 4 to pH 6. The skin pH is influenced by endogenous factors, including skin moisture, sweat, race or anatomic site, or by exogenous factors including soaps, detergents or other skin irritants [Yosipovitch et al. (1996), Schneider et al. (2007)]. The internal body pH usually has a value between 7.35 and 7.46 [Yosipovitch et al. (1996)]. The pH of the skin is fairly stable throughout a lifetime. The pH rises slightly after approximately the age of 80 or with diabetes development. An alkaline pH is meant to weaken the barrier function of the skin and therefore promotes possible skin infections [Yosipovitch et al. (1996), Schneider et al. (2007)].

The pH on the skin is believed to be controlled by acid production, including amino acids and lactic acid, and the consistency of the secreted sweat in which the concentration of ammonium and hydrogen can vary [Yosipovitch et al. (1996), Schneider et al. (2007)].

2.3.c pH in Normal, Acute and Chronic Wounds

The exact pH change during wound healing is not completely understood yet, but generally it can be said that normally healing wounds have a pH of 5-9 while chronic non-healing wounds and bacterial infected wounds have an alkaline pH exhibiting a different pattern instead of above with at least 7.3 [Schneider et al. (2007), Ono et al. (2015)].

Shown in figure 10 and figure 11 are the pH changes of acute and chronic wounds respectively after injury. In the graphs it can be seen that acute wounds show a drop to an acidic pH, followed by a rise in pH just before re-epithelisation during which the pH declines to an acidic pH again [Schneider et al. (2007)].

The chronic wound pH process, on the other hand, has a small drop in pH just after the healing stage which is followed by a drastic rise in pH to alkaline levels. The chronic wound pH will stay at alkaline pH relatively stable with small fluctuations for several months or even years [Schneider et al. (2007)].

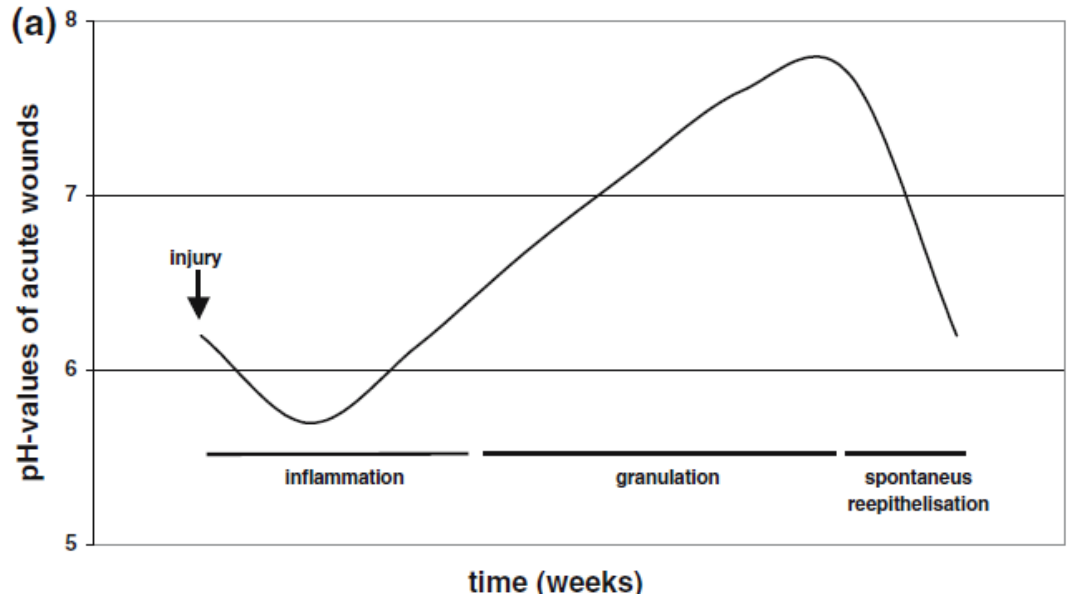


Figure 10: pH change of an acute wound during the healing process. After a drop into acidic milieu the pH rises to alkaline pH before it drops again during re-epithelisation after a few weeks [Schneider et al. (2007)].

The drop in pH can partly be explained by a host innate defence mechanism in which foreign material and bacteria are combated by neutrophils. Also contributing to the acidification of

the wound is the generation of organic acids linked to the increased collagen production, an increased demand for O_2 and local pCO_2. A causal relationship between change in wound pH and bacteria presence or even bacteria species identification has not yet been established [Schneider et al. (2007)].

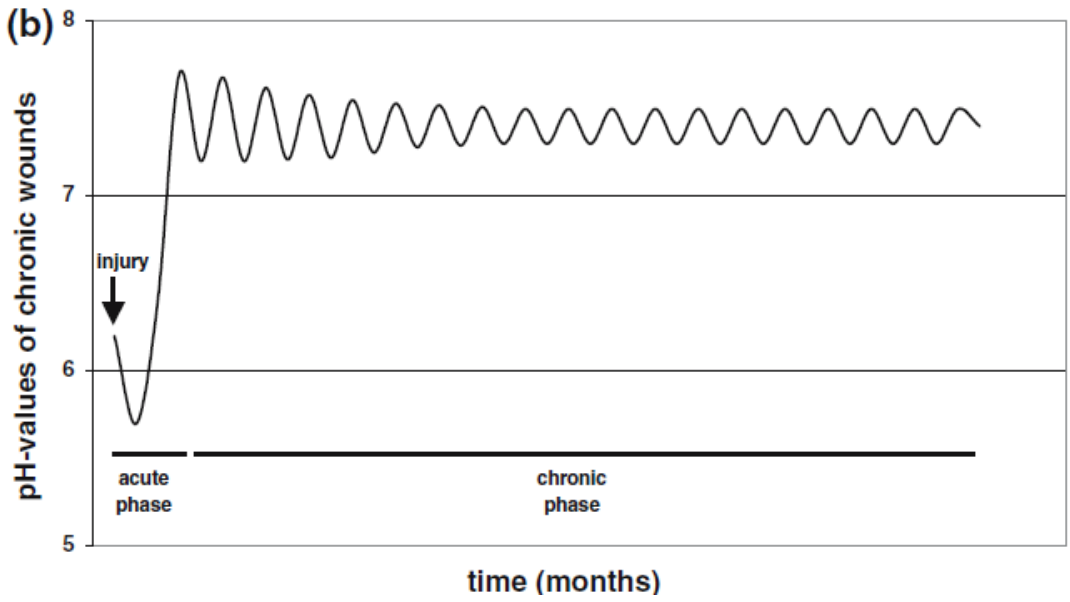


Figure 11: pH change of a chronic wound during the healing process. After a drop into acidic milieu the pH rises to alkaline pH where it will stay at an almost stable pH with small fluctuations for months or even years [Schneider et al. (2007)].

Figure 12 shows the pH values of wound fluids taken from two pig wounds during healing from a study conducted by Shi et al. (2011). Generally the wound pH stays alkaline until the wound starts healing and the pH drops steadily.

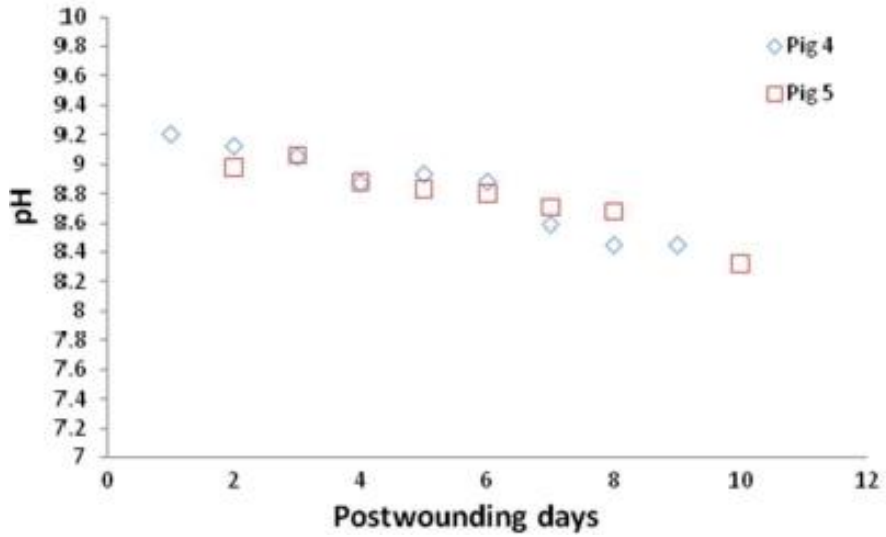


Figure 12: Change in pH in wound fluid of infected pig wounds during healing. It is noted that the wound pH was highly alkaline and dropped to a lower alkaline pH during healing [Shi et al. (2011)].

Wilson et al. (1979) conducted a study in which 50 patients with chronic leg ulcers had a mean pH of 7.7 (±0.3 SD) with a total range of 7.3-8.9 [Wilson et al. (1979)]. Other studies confirm these findings and also reveal that a healing chronic wound during re-epithelisation has a lower pH of 6.0 (±0.5 SD) [Schneider et al. (2007)].

The wound pH is historically neglected and only assumed that an acidic pH is favourable for all wound healing. It has however, been shown that, even though a low pH is favourable for certain healing stages, a high pH value is correlated to improved take-rates of skin-grafts. For chronic wounds with pH values below 7.4, the take-rate for skin-grafts is almost none while the take-rate for wounds with a pH higher 7.4 is 99% [Schneider et al. (2007)].

2.3.d Importance of pH in Wound Healing

The pH within a wound is a very important factor during wound healing. It is influencing directly and indirectly all reactions in the wound bed area [Schneider et al. (2007)].

Healing wounds have a higher oxygen demand than normal skin to fuel all required reactions in the wound site. The so-called Bohr-effect is responsible for higher O_2 supply to the wound than normal. The basis of the Bohr-effect is the fact that the binding capability of haemoglobin to oxygen is decreased for environments with decreased pH (i.e. lower than the physiological level of 7.4) or for increased CO_2 levels in tissue. A healing wound has an acidic pH and will therefore receive more oxygen than a non-healing chronic wound with an alkaline pH [Schneider et al. (2007), Trengrove et al. (1999)]. An increased pH in turn will directly limit the healing capabilities of a wound by limiting oxygen supply.

The effectiveness of antibiotics decreases drastically with decreasing pH [Schneider et al. (2007)]. This further complicates treating bacterially infected wounds. It has been shown that the activity for the aminoglycoside gentamycin in an environment with pH of 7.8 has a 90-fold higher biological activity than in an acidic environment of pH 5.5 [Mehmood et al. (2014), McArdle et al. (2014) and Schneider et al. (2007)].

Moreover, the performance of antiseptics is also impaired by pH changes. The working efficacy of silver for example is impaired by increased pH because the solubility of metal ions is decreased [Percival et al. (2014)]. The use of other antiseptics with a working range in the alkaline levels is suggested for chronic wounds [Percival et al. (2014)].

Matrix metalloproteinases (MMPs) are enzymes in wounds responsible for the degradation of dead tissue as a part of wound healing and repair but may cause destruction of key molecules including growth factors [McArdle et al. (2014), Gethin et al. (2008)]. In chronic wounds this enzyme has been found to be overrepresented [Muller et al. (2007)]. This will lead to a faster tissue breakdown than synthesis which keeps the wound in a prolonged

inflammatory phase. Furthermore, it has been found that the enzyme inhibitor of matrix metalloproeases (TIMPs) is underrepresented in chronic wounds, amplifying this effect [McArdle et al. (2014), Schneider et al. (2007) and Muller et al. (2007)].

All enzymes have an ideal working pH in which its activity reaches a peak. It is however, possible for the enzyme to work a defined range around that ideal pH. Outside the optimal pH the enzyme will change its three-dimensional structure and lose its functionality [McArdle et al. (2014), Schneider et al. (2007) and Trengrove et al. (1999)]. In figure 13, a list of enzymes used in wound debridement is presented with their pH working range [Schneider et al. (2007)]. MMPs are inactivated at an acidic pH and reaches their peak activity at higher pH values [McArdle et al. (2014)]. It should be noted that not only more MMPs are present in chronic wounds, but that the pH environment also supports their activity which leads to further degradation of the wound tissue without sufficient tissue reassembly leading to further delayed healing [Schneider et al. (2007), Muller et al. (2007) and Gethin et al. (2008)].

DNAse
Activity pH 4.5–5.5 (fibrinolysis)
Activity pH 7.0–8.0 (DNA lysis)
Fibrinolysin
Activity pH 7.0–8.0
Kollagenase
pH optimum 6.0–8.0
Krill-enzyme
pH optimum above 7.5
Papain
Activity pH 3.0–12.9
pH optimum 7.0
Plasmin
pH optimum 7.0
Streptodornase
pH optimum pH 7.5
Streptokinase
Activity pH 7.3–7.6
Sutilain
pH optimum 6.0–6.8
Trypsin
pH optimum 7.0

Figure 13: Enzyme peak activity used for wound debridement [Schneider et al. (2007)].

During bacterial metabolism it is common to produce ammonia as end product that is subsequently released into the environment. Ammonia can damage the wound tissue and its histotoxicity is directly correlated to the environmental pH in the wound bed. The toxicity towards the tissue is the strongest in slightly alkaline pH [Gethin et al. (2008)]. It should be noted that *S. aureus* is suspected to be able to use free amino acids and cellular proteins as endogenous substrate to produce ammonia in the supernatant [Ramsel et al. (1961)].

Furthermore, the body's immune system is affected by the wound fluid pH [Schneider et al. (2007), Gethin et al. (2008)]. Not only are important wound healing enzymes eventually inactive because of a shifted pH range, but the proliferation rate and activity of fibroblasts and macrophages is also influenced by the surrounding pH [Gethin et al. (2008)]. An acidic

environment is important for macrophage recruitment to the wound bed [Percival et al (2014)]. Furthermore it was found that macrophages cultured in low pH environments failed to produce angiogenic factors which will further impact wound healing [Percival et al. (2014)]. In a study using impermeable wound dressings to lower the pH of the chronic wound to slightly acidic milieu it was found that the lowered pH promoted fibroblast growth and had the capability to inhibit bacterial growth [Schneider et al. (2007)]. High pH values in the alkaline level also increase apoptosis and decrease locomotion of polymorphonuclear leukocytes (PMNs) and neutrophils [Percival et al. (2014)].

It is generally understood that pathogenic bacteria need pH values above pH 6 to be able to growth uninhibitedly [Schneider et al. (2007)]. *S. aureus* is also known to withstand acidic pH values as low as 5 pH before it is affected by it [Percival et al. (2014)].

2.3.e Current pH measurement techniques and their limitations

Glass electrodes are the gold standard measurement technique to record pH values. They consist of an H^+ sensitive electrode and a reference electrode that is proton concentration independent. With the help of a potentiometer the pH in the sample solution can be detected by comparing the data from both electrodes. However, it is not possible to use glass electrodes reasonably in chronic wounds or any environment that offers opportunity for bacterial contamination. Furthermore, it is rather difficult to ensure complete sterilisation and normal working of the glass electrode. Moreover, the sample size suitable for glass pH meters has to be big enough to cover the sensing part of the device. In chronic wounds however, the sample that can be drawn is often very small and not enough for a glass electrode pH measurement. Additionally it is not given that this sensing technology can be

miniaturised any further. For a successful implementation of pH sensing wound dressing, however, it would have to be miniaturised and simplified, which is both not yet possible. Glass is brittle and doesn't deform which makes it unsuitable for wearable monitoring in a wound. For all these reasons a different pH measurement system was used for this thesis [Mahon et al. (2015)].

As mentioned before in section 2.1.d Current Bacteria Detection Techniques, the most common way to sample a chronic wound is by a swab. The biosensor used in this thesis is applicable with the swab technique. Hereby a swab is taken with a cotton bud which is rolled over the two electrodes on the sensor after calibration. This unproblematic integration in a clinical setup and, moreover, ease of integration to a wearable pH sensing wound dressing was crucial for the decision of using this type of sensor [Murray et al. (2009), Burmølle et al. (2010), Mahon et al. (2015)].

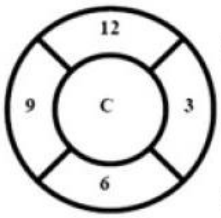
2.3.f The Need for Innovation

Chronic wounds are a great burden to health care systems all over the world. Subjective analysis of signs and symptoms are not accurate enough to detect bacterial infections early and microbiological tests take too long and cannot analyse the entire wound site at the same time [Murray et al. (2009), Burmølle et al. (2010) and Fazli et al. (2009)].

The major limitation of current bacterial detection methods, introduced in section 2.1.d Current Bacteria Detection Techniques, however, is the highly heterogeneous bacteria distribution in chronic wounds shown in figure 14 [Burmølle et al. (2010), Fazli et al. (2009)]. The bacteria population varies on the wound surface and depending where in the wound the samples were taken, the detected bacteria will change. This was shown in several studies,

from Fazli et al. (2009) and Wolclott et al. (2009), and underlines the importance of a more accurate diagnostic method [Burmølle et al. (2010)]. Furthermore, Thomsen et al. (2010) showed that the bacteria distribution within the wounds follows a general pattern where *S. aureus* is mostly located on the surface of a chronic wound while *P. aeruginosa* is located in deeper tissue areas. Q-PCR is a fast identification method, but it is not possible to distinguish between living and dead bacteria [Burmølle et al. (2010), Thomsen et al. (2010)].

(c)



	Method	*S. aureus* Wound E	*S. aureus* Wound F	*P. aeruginosa* Wound E	*P. aeruginosa* Wound F
C	q-PCR	89±11%	200±13%	510±18%	920±9%
	Cultivation			+	
	DGGE	+	+		
3	q-PCR	No sample	86±8%	No sample	300±13%
	Cultivation				
	DGGE		+		
6	q-PCR	240±10%	290±8%	760±7%	8200±8%
	Cultivation			+	
	DGGE	+	+		
9	q-PCR	310±13%	80±5%	47±9%	800±10%
	Cultivation			+	
	DGGE	+	+		
12	q-PCR	180±8%	93±12%	280±3%	15±5%
	Cultivation			+	
	DGGE	+	+		

Figure 14: This shows the high heterogeneous bacteria distribution within a wound site. The circle on the left shows the geographic location of the swap and the table on the right shows the amount of bacteria present in this location for wounds E and F for S. aureus and P. aeruginosa [Burmølle et al. (2010).

Additionally, the wound cover has to be removed to conduct a visual inspection or take a sample. This produces stress for the wound milieu and pain for the patient and should therefore be avoided if possible. An *in vivo* or at least in bandaged integrated measurement technique to asses bacterial infection has to be developed [Schröter et al. (2012)].

The changing pH of chronic wounds can be used as a basis to detect bacteria presence in a wound site. This is however, not yet established. It is possible to integrate miniature pH sensors into bandages in order to produce wearable and cost efficient pH sensors. These

could be read out or record data continuously and would be able to detect bacterial infections as soon as possible. This has the potential to avoid long wound healing and prevent antibiotic misuse and reduce overuse. The ideal sensor is cheap, easy to apply, disposable, flexible, robust, sterilisable and integrated into the bandages [Schröter et al. (2012)].

In the future it is also possible to integrate several sensors into the wound bandaged to assess several important wound healing parameters. Possible measured parameters are moist of the wound, temperature, and pH.

2.3.g The Disposable Sensor and its Working Principle

The sensor used in this thesis was developed and produced in the biomedical Engineering Department of Biomedical Engineering at the University of Strathclyde, Glasgow. Figure 15 illustrates the general setup of the disposable sensing compound.

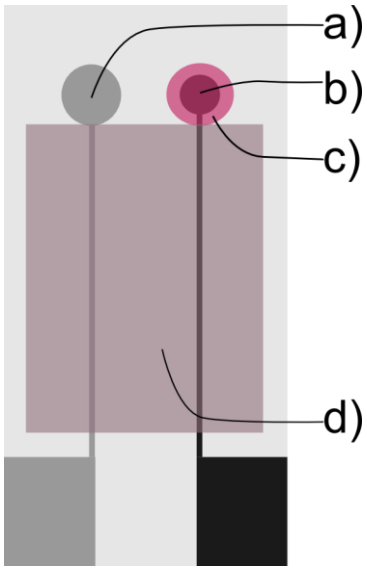


Figure 15: Schematic of the disposable pH sensor; a) shows the Ag/AgCl reference electrode, b) the working electrode while c) labels the semi permeable membrane applied to the working electrode and d) the electrode insulation to protect the sensor from getting wet and therefore change the output.

It is composed of a reference electrode made out of Ag/AgCl and a working electrode out of carbon. Both electrodes are applied via screen printing. On the working electrode, a layer of selectively permeable membrane is applied and the connecting electrode pathways are insulated using self-adherent foil to prevent them to wetting or damage during the working procedure.

The working principle of the sensor is based on its potentiometric measurement capability. Per definition it is known that the higher the pH, the lower the H^+ concentration in the sample solution. The permeable membrane on the working electrode is selective to protons and allows them to migrate into the membrane. The carbon electrode is H^+ sensitive and its measured potential will change with the concentration of protons present. The reference electrode on the other hand will not change potential with proton presence and using this the pH can be calculated.

The disposable sensor is connected to a portable measurement device and calibrated as described in section 3.7 pH Measurement. The voltages for both calibration solutions, pH 4 and pH 7, are recorded and an approximately linear system correlating measured voltages and pH is developed for this individual sensor. One example of a linear correlation of voltage and pH for a range of pH 4 to pH 10 can be seen in figure 16.

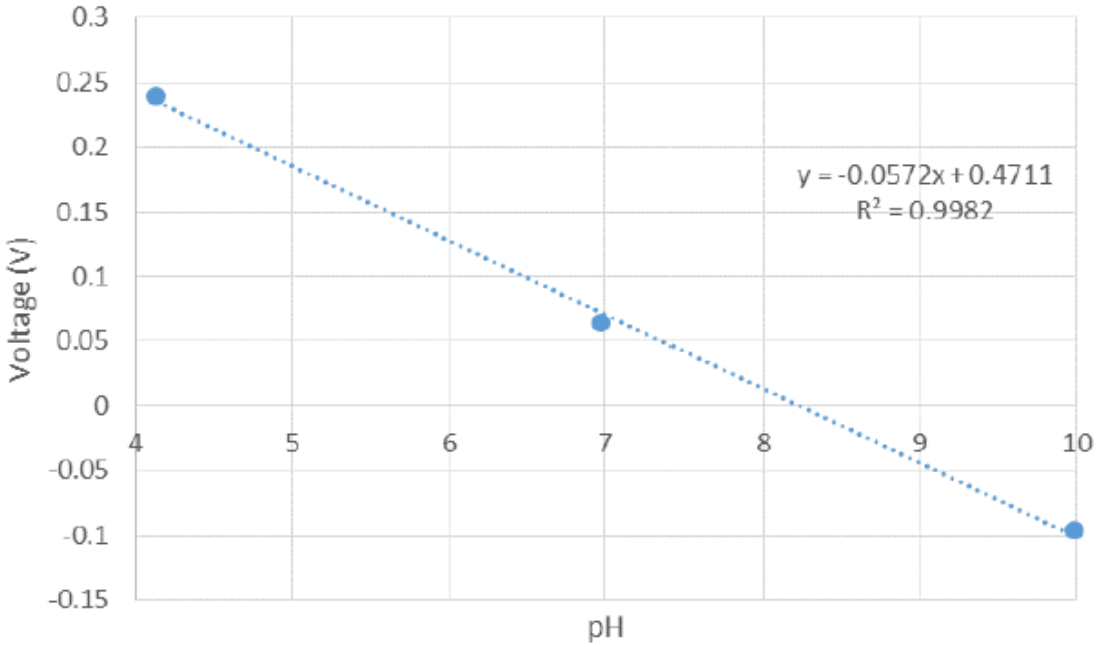


Figure 16: Linear correlation between measured voltage and pH for a pH rang of pH 4 to pH 10 [Milne et al. (2014)].

Using linear fitting, the slope, m and offset, c between the pH and potential can be found.

The bases of the calculation are these two equations:

$$m = \Delta V / \Delta pH$$

$$c = V_4 - m * pH_4$$

Where m is the calculated slope, ΔV is the difference in voltage between the two samples ΔpH is the difference in pH of the samples, c is the offset of the fitted curve, V_4 is the recorded voltage for the pH 4 calibration solution and pH_4 is the pH of the calibration solution pH 4 which is 4.00 pH.

Every sample will yield a specific voltage. This voltage will be used together with the previously calculated slope, m and offset, c of the system to reconstruct the pH of the sample solution. The formula used to calculate the pH is given below:

$$pH_s = (V_s - c)/m \ ,$$

where pH_s is the reconstructed pH of the sample and V_s is the recorded voltage of the sample.

2.4 Literature Summary

All in all it can be noted that chronic wounds are a big burden on society and health care systems all over the world. The exact reasons for chronic wound development and delayed wound healing are inconclusive and an additional risk are chronic wound infections with bacteria. Bacterial metabolism is highly complex and can adapt to changing environmental conditions. While the pH in normally healing wounds is mostly acidic, the pH in chronic wounds is alkaline which results in decreased antibiotic efficiency, shifted enzyme activity, decreased fibroblast activity and less oxygen supply to the wound site. Bacteria presence has been shown to increase the wound pH as well but no study has sufficiently investigated the pH change of infected chronic wounds.

With the help of a disposable pH sensor that can be integrated into wound dressings in the future, the pH of *S. aureus* and *S. epidermidis* are investigated *in vitro* in LB media and FBS. The aim is to determine the feasibility to detect bacteria presence in chronic wounds according to its pH change. Once this is approved, further studies can be conducted using multi-parameter arrays to determine bacterial infection in chronic wounds as early as possible.

3. Methodology

Materials and methods used in this thesis are described in this section. The type of bacteria, media and calibration solutions are stated as well as the procedures of production and calibration of the disposable sensor and how to conduct a colony count. Finally the precise experimental setup is described with the processes and measurements made.

3.1 Bacteria

For this thesis the bacteria under investigation were *S. aureus*, NCTC 10788, and *S. epidermidis*, NCTC 11964. From the data sheet of the National Collection of Type Cultures it is known that the optimal growth conditions for these facultative anaerobes is 37°C in nutrient broth [Public Health England (2013), Public Health England (2013)].

3.2 Media

Three main media types were used to grow and compare the two bacteria *S. aureus* and *S. epidermidis*; Luria Bertani (LB) media, mannitol salt agar (MSA) and foetal bovine serum (FBS). LB and MSA were poured into solid agar plates which in turn were used for colony counting procedures and maintaining stock cultures of the bacteria. Liquid media on the other hand was used for preparation of serial dilutions, growth curve experiments and overnight cultures.

3.2.a LB Media

Media was made by mixing 10g of tryptone (order number: 1279-7099, Fisher Scientific), 5g NaCl (order number: S7653, Sigma-Aldrich) and 5g yeast extract (order number: BP1422-100, Fisher Scientific) with 1l of deionised H_2O. If solid media was required, 5% agar (order

number: BP1423-500, Fisher Scientific) had to be added to the solution as well. Afterwards the media was autoclaved for 20 minutes at 121 C. Media plates were poured in a laminar flow hood and once the media cooled to approximately 50°C. Plates were left to dry for at least 20 minutes and were then stored at 7°C prior to use.

3.2.b Mannitol Salt Agar

To make MSA, 111g of already prepared Mannitol Salt Agar (order number: CM0085, Oxoid) in power form was mixed with deionised H_2O to produce 1l of media. After mixing it thoroughly, the media was autoclaved for 20 Minutes at 121 C. It is important to know that MSA contains phenol red and can therefore indicate pH changes in the media.

3.2.c Foetal Bovine Serum

In order to simulate chronic wound fluid conditions, Foetal Bovine Serum (order number: 10500-064, Gibco®) was used for a growth curve experiment and long term testing. The FBS is from an E.U.-approved origin, South America, and was bought heat inactivated. Milne et al. (2014) used foetal horse serum as an *in vitro* model of wound fluid which yielded comparable results to the *in vivo* samples from wound sites. Foetal horse serum is physiologically very similar to foetal bovine serum and therefore justifies its use as an *in vitro* model for immunosuppressed chronic wounds [Milne et al. (2014)].

3.3 Calibration Solutions

In order to calibrate the disposable pH sensors, calibrations solutions had been produced. Due to the fact that wound fluid and LB media contains sodium chloride which shifts the working values of the sensor, the calibration solutions had to be adapted to the salt concentration contained in the sample probes. The buffer solutions described below were

adjusted to have a pH in the range of pH 2-11 with an ionic strength range between 0.007-0.1.

3.3.a Acidic Buffer

Due to the ingredients needed for this buffer, the entire procedure was performed under a fume hood. First of all, 900ml deionised water was poured into a 1l glass bottle. Second, the following ingredients were accurately measured, added to the bottle and ensured that the content is properly mixed; 2.3ml Acetic acid (order number: 320099, Sigma-Aldrich), 2.74ml Phosporic acid (order number: 04102, Sigma-Aldrich) and 2.4732g Boric acid (order number: B6768, Sigma). Finally, enough distilled water was added to fill the content to exactly one litre of liquid.

3.3.b Alkaline Buffer

A 1 litre bottle was filled with 875ml distilled water and 7g of NaOH (order number: S5881, Sigma-Aldrich) added and mixed thoroughly to make a 0.2M NaOH solution.

3.3.c Chloride Adjusted Buffer

The disposable printed sensors were calibrated before every use. The calibration solution had to be chloride adjusted in order for the sensor to measure the correct pH vales.

About 500ml of acidic buffer solution was added to a glass beaker and the amount of sodium chloride added to mimic probe environmental conditions. A glass electrode was continuously measuring the pH while small amounts of sodium hydroxide were pipetted into the beaker until the desired pH is achieved. This was done to produce a chloride adjusted pH 4 and pH 7 calibration solution.

3.4 pH Indicator

PH indicators are useful to estimate the approximate pH value of a solution as a control to the pH electrode.

3.4.a Phenol Red

Useful pH range of phenol red (order number: P0290, Sigma) is between 6.8 and 8.2 in which it will indicate a red colour. Below 6.8 the colour is bright yellow and above 8.2 the colour is strongly pink [Mahon et al. (2015)]. In this study it is used as a control to the disposable pH sensor in the growth curve experiments.

3.5 Disposable pH Sensor Production

The production of the senor is described below. In figure 17, the production process of the disposable sensor is illustrated. Firstly, the reference electrode was printed on a plastic substrate using screen printing technology. The reference electrode was made of Ag/AgCl, which was the reason the produced pH sensor was chloride sensitive and the calibrations solutions had to be chloride adjusted according to the expected salt levels of the samples.

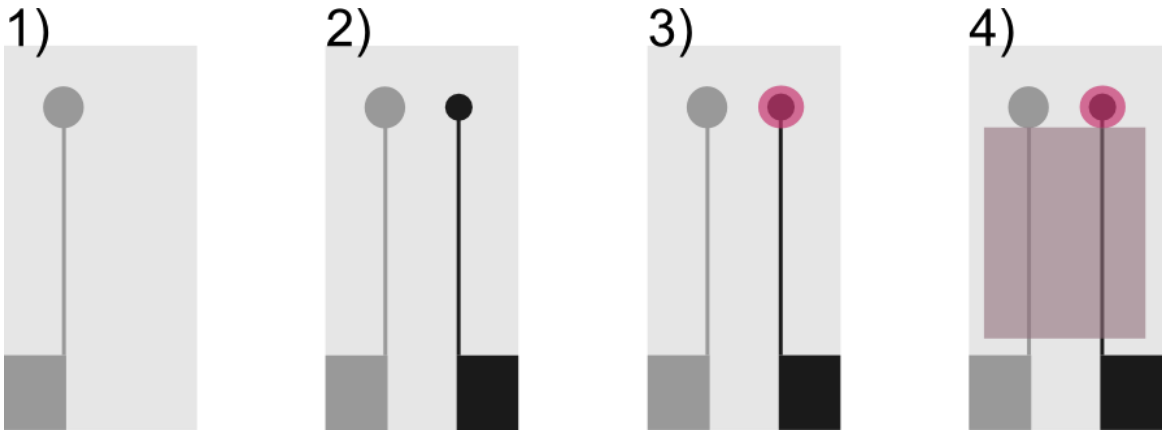


Figure 17: Sensor production explained step by step. In step 1) the Ag/AgCl reference electrode is screen printed on the plastic wafer. With the same process the working electrode is printed out of carbon in step 2). In step 3) a selectively permeable membrane is applied to the working electrode and finally, in step 4) both electrodes are insulated to avoid alteration of the output by, for example, a wet electrode.

Secondly, the carbon working electrode was also screen printed onto the same substrate. It was important that the distance between the two electrodes is not too large because the applied sample had to be able to touch both electrodes at the same time. The preferred distance between electrodes is about 5 mm. This allows sample volumes as low as 10 µl.

Thirdly, a selectively permeable membrane was applied in liquid form onto the working electrode and air dried. This membrane was necessary for the sensor to only detect pH changes and not to be affected by other ion concentration changes. It has been shown that the sensor is stable to Na^+, K^+ and other small molecules [Milne et al. (2014)].

One common problem was the electrode-meter contacts which leads to unstable measurements. Another problem occurring less frequently was a poor connection due to scraped off ink by the connector. Once the connection between the electrode and the measurement device was disrupted, no more measurements could be conducted. For all

these reasons the printed sensor was insulated by a self-adhesive plastic to prevent measurement alterations.

The disposable pH sensor is attached to a measurement device shown in figure 18. After calibration and sample application, explained in more detail in 3.7 pH Measurement, the measured voltage and corresponding pH is displayed in the small monitor of the box.

The working principle of the sensor is explained in more detail in 2.3.f The Disposable Sensor and its Working Principle.

Figure 18: This shows a typical pH measurement. The disposable sensor is attached to the measurement device on the top. After calibration the integrated monitor shows the measured voltage and the corresponding pH of the applied sample.

3.4 Colony Counting

To determine the bacteria density in the media at a certain time point, a sample was taken from each of the replicates incubated with bacteria. The entire process was executed under a laminar flow hood.

Eight wells were prepared with 180µl LB media in a 96 well plate (order number: 92096, TPP) using a multichannel pipette. In figure 20 the process is illustrated with fifth dilution. In the first well 20 µl of the overnight incubated culture media was added and sufficiently mixed with the help of a pipette. Using a fresh pipet tip, 20 µl of the first well solution was transferred to the second well. This procedure was repeated until the last well was inoculated. This serial dilution produces a logarithmic change of concentration between the dilutions.

The chosen dilutions were then plated on an LB agar using the drop plate method [Herigstad et al. (2001)]. In this method, ten 10 µl drops were placed onto an agar plate for every dilution (making a total plated volume of 100 µl). The same was done for two other dilutions until a total of thirty drops were dispensed on the agar plate.

All agar plates were air dried, incubated overnight for approximately 15 hours. Two typical colony counting plates of *S. aureus* on the left and *S. epidermidis* on the right are shown in figure 19 and the colonies per drop can be counted. The total of colonies counted of the chosen dilution should be between 30 and 300, i.e., every drop should contain between 3 and 30.

The total colony count of one dilution is called the CFU per μl. To get the more comparable CFU/ml, the results will have to be multiplied by 10/Dilution Factor.

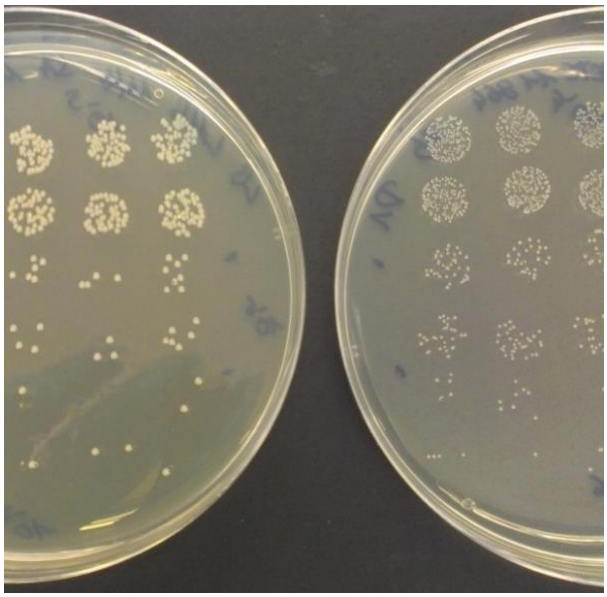


Figure 19: The LB plate on the left shows a colony count of S. aureus while the one on the right shows three plated dilutions of S. epidermidis solutions.

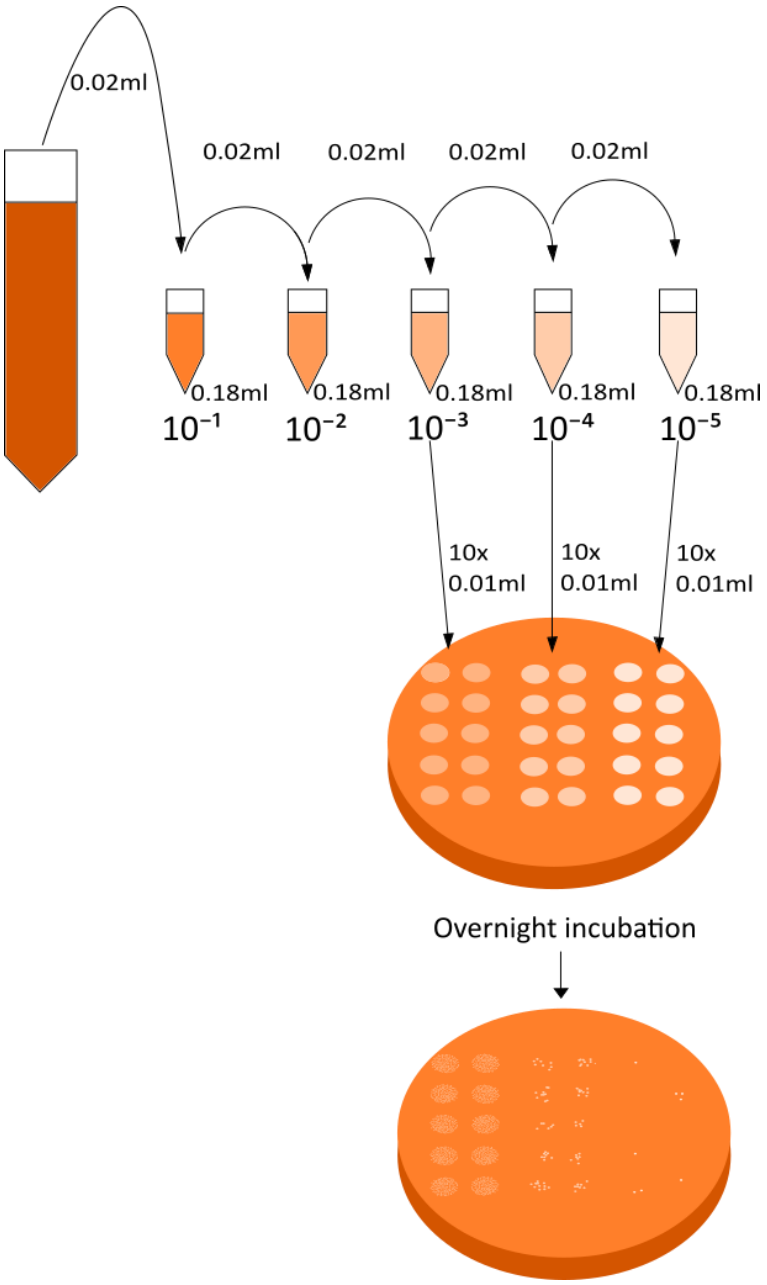


Figure 20: Illustrates the serial dilution and droplet plating arrangement on the agar plate. Firstly, 0.02ml of overnight incubated media is inserted in 0.18ml fresh media. Secondly, 0.02ml of the first dilution is inserted in a second container with fresh media. This process is repeated until the desired dilutions are produced. The three desired dilutions are plated in ten 0.01ml drops each on an agar plate, air dried and incubated overnight. The amount of produced colonies can be counted the following day and the CFU/ml calculated. The drops of the third (10^{-3}) dilution exhibits too many colonies to count, while dilution 10^{-5} shows only a total number of colonies of 8. Both dilutions don't qualify to be used to calculate the Colony Forming Unit (CFU) per ml. The dilution of 10^{-4}, however, shows a sufficient number of colonies per drop and is recorded and used for further calculations.

3.7 pH Measurement

For each pH measurement a new disposable pH sensor was used after following the calibration procedure described below. The minimal volume of liquid that can be used to measure the pH of the sample or to calibrate the sensor is 10 µl. The calibrations process is illustrated in figure 21.

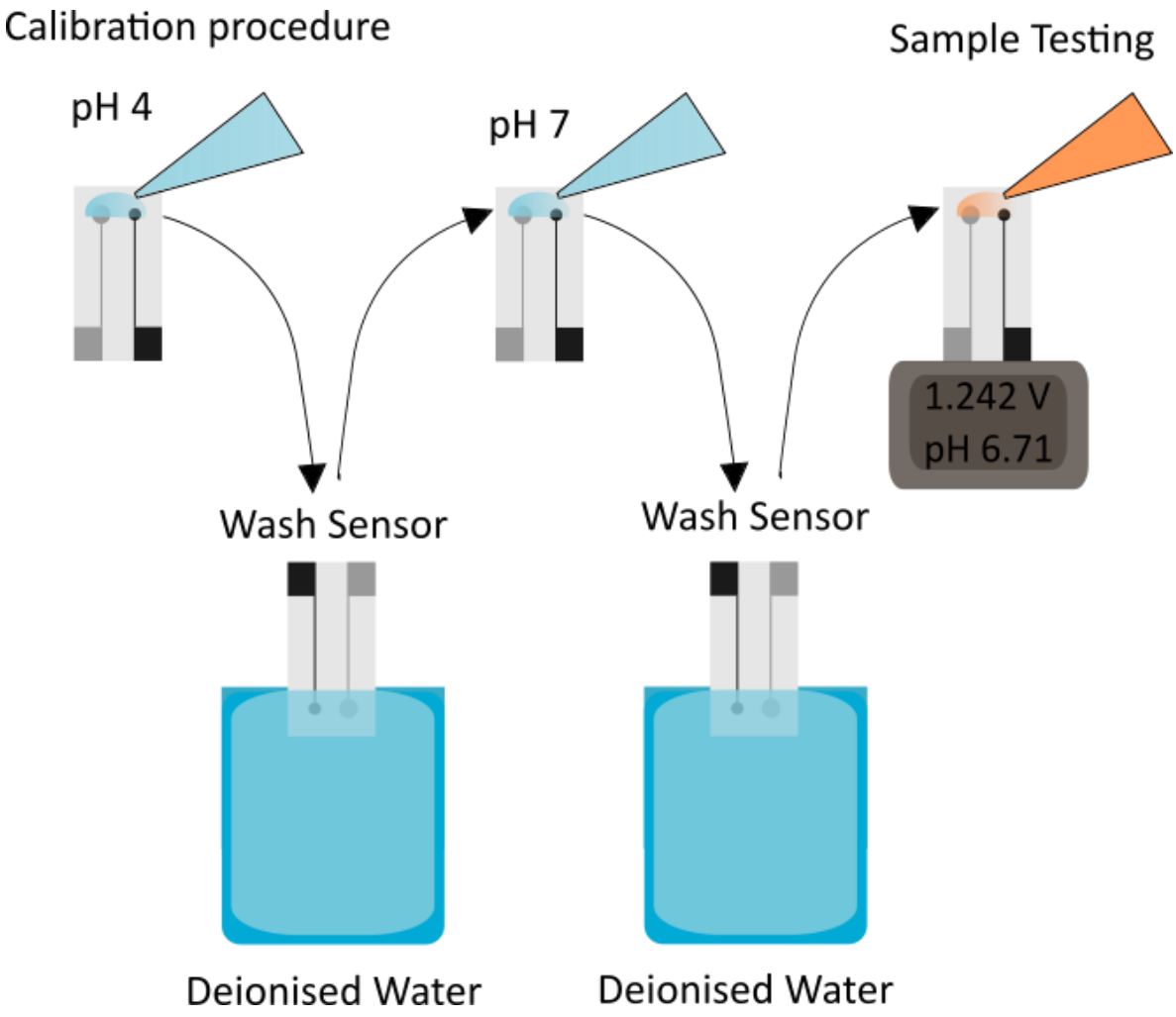


Figure 21: The pH sensing processes illustrated in this graphic. First, a 0.015ml drop of pH 4 calibration solution is applied to the sensor. After the voltage value has settled, a button is pressed and the sensor washed. Second, a 0.015ml drop of pH 7 calibration solution is applied and washed again after the voltage has settles again and the button has been pressed. Finally, a 0.015ml drop of sample solution is put on and the Voltage and pH can be read out.

A drop of chloride adjusted pH 4 calibration solution was applied to the sensor. Once the measured voltage was stable, a button was pressed to save the value and the sensor was repeatedly dipped in deionised water to remove the calibration solution. The excess solution was removed with blue wipe and a drop of chloride adjusted pH 7 calibration solution was applied to the sensor. The stabile voltage level was saved with a second press on the button and the sensor was washed repeating the previously described procedure. The sensor was than fully calibrated and ready for use. A drop of the sample solution was applied to the sensor and after the measured voltage and pH had settled the values were recorded and the sensor was washed as described above and used for the next probe measurement. The sensor was disposed in the autoclave bin after the measurements and the wash solution, i.e. the deionised water, was disposed the same way within the 25 ml universal container it was stored in during the experiment.

3.8 Bacteria Characterisation

It was necessary to predict growth rate and roughly estimate bacteria counts to aid experiment planning and execution. To achieve this, bacteria characterisation experiments were performed.

Eight wells of a 24-well-plate were filled with $1\,ml$ of LB media and four were inoculated with *S. aureus* and *S. epidermidis* accordingly. Furthermore, a universal container (DNN41804, Elkay Laboratory Products (UK) Lt.) with $5\,ml$ was also inoculated with one of the two bacteria. The two universals were placed in a shaking incubator overnight for about 15 hours at 37°C while the 24-well-plate was placed in a non-shaking incubator overnight for about 15 hours also at 37°C. A colony count was done the following day.

A colony of *S. aureus* was streaked out on a MSA plate and placed in a non-shaking incubator at 37°C overnight. The same was done for *S. epidermidis* and both plates were compared the next day.

3.9 pH Sensor Measurement Verification of LB Media

The disposable sensor has been shown to measure the pH of wound swaps in the hospital successfully using the chloride adapted calibration solutions [Milne et al. (2014)]. In order for the sensor to be suitable for experiments with LB media, appropriate verification tests have to be conducted.

Liquid LB media only consists of three ingredients: sodium chloride, tryptone and yeast extract. Sodium chloride in LB media and wound fluid is known to affect the reference electrode of the sensor which is why the calibration solutions were adapted accordingly.

An experiment investigating the influence of the other ingredients on the working or reference electrode was executed. In Part A of the experiment three different concentration solutions of tryptone and yeast extract were prepared in deionised water. The concentrations were chosen according to their concentration in the media, half of this concentration and double this concentration. In Part B sodium chloride was added to the solutions. The theory is that sodium chloride will dominate the behaviour and therefore stabilise the sensor's voltage output. In Part C the output of both electrodes to LB media and both chloride adjusted electrodes is measured.

All solutions were measured with the working electrode and the reference electrode each compared to the measurements of a glass electrode. The glass electrode and one part of the

printed sensor was attached via a Crocodile clip to a Solartron electrochemical interface 1287A and immersed in the sample solution. The voltages were recoded with the help of a computer and were later compared to each other.

3.10 pH Measurements in Bacterial Cultures

The experiments done to investigate the correlation between pH and bacterial growth for *S. aureus* and *S. epidermidis* are discussed in this section. At first a 25 hour long growth curve experiment was implemented in LB media (figure 22). During this experiment the general pH variation behaviour was investigated for both bacteria in comparison to each other using four repetitions. Furthermore, this experiment was repeated in FBS (figure 24). Heat inactivated FBS was used as a model for immunosuppressed chronic wound fluid environment and was the cheapest and most similar *in vitro* media available to mimic these conditions. A growth curve experiment over 25 hours and, moreover, a long term growth experiment over 72 hours was conducted in FBS with both bacteria using four iterations (figure 25). This way the pH profile during the log phase could be investigated in more detail and the general pH change during all four bacterial growth stages was observed for both bacteria.

3.10.a Growth Curve Experiment in LB media

To investigate the bacterial growth and pH change of the media, a growth curve experiment in LB media was implemented. A universal container with 5 µl LB media and three colonies of either *S. aureus* or *S. epidermidis* was prepared and incubated overnight at 37°C. A general overview of the experiment is illustrated in figure 22.

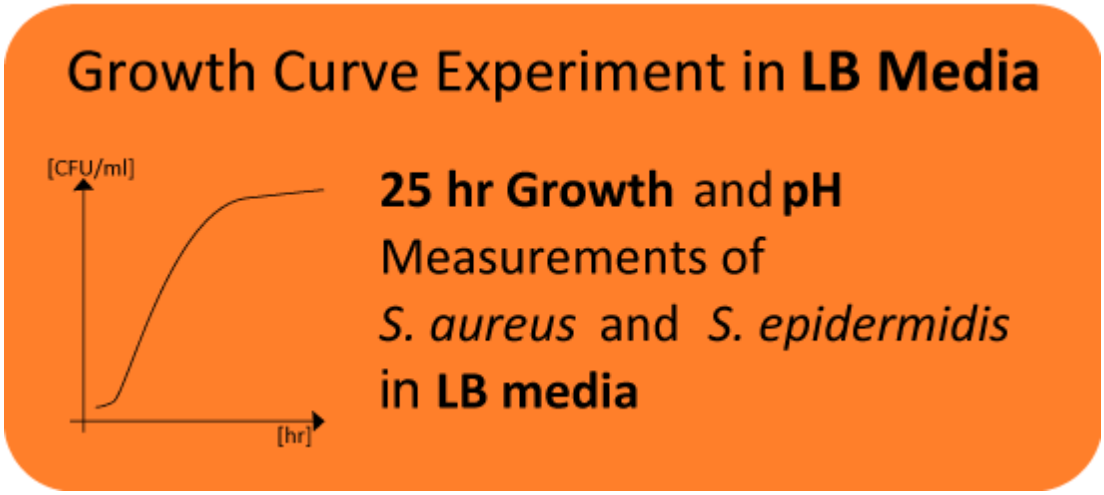


Figure 22: Growth Curve Experiment in LB. This shows the measurements done in LB media over 25 hours. The expected outcome of the bacterial growth during that time is given on the left.

A 24-well-plate (order number: 114642, Delta, Nunclon Surface) was prepared for the experiment as shown in figure 23. All wells were filled with $1\,ml$ of liquid. 12 wells were filled with LB media and 12 wells with phenol red coloured LB media which was used as a control to check the consistency of the disposable pH sensor as described in 3.4.a Phenol Red. Four of the wells containing LB media and four of the wells containing phenol red coloured LB media were inoculated with 10 µl of the overnight bacteria culture of *S. aureus* while another four wells of each were inoculated with *S. epidermidis*. The remaining four wells with LB media and phenol red coloured LB media served as contamination controls.

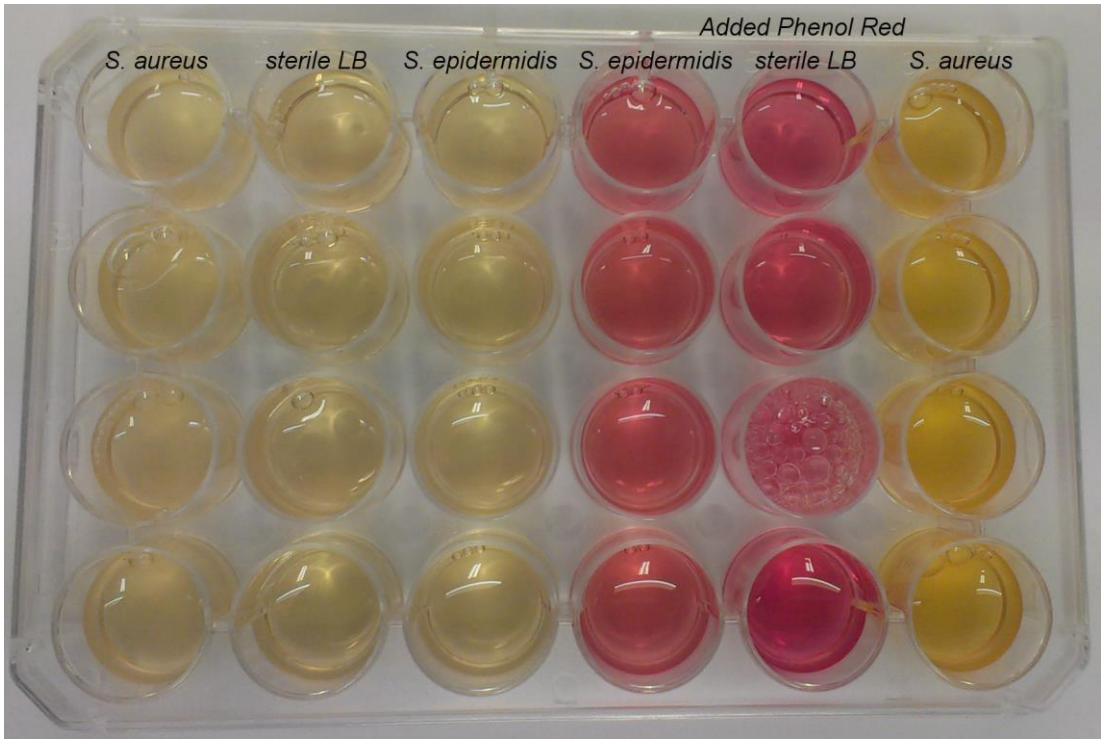


Figure 23: 24-well-plate setup of the Growth curve experiment in LB media. The three columns on the left were filled with 1 ml LB media and the three on the right were filled with phenol red coloured LB media. The two outer most columns were inoculated with S. aureus, while the two middle columns were inoculated with S. epidermidis. The second and fifth column serve as negative controls. The third well in this column is filled with bubbles due to accidently pipetting air into the media. This picture was taken four hours after the start of the experiment which is why a colour change in the S. aureus inoculated phenol red control is visible.

The first measurement was taken straight after the inoculation of the wells and every hour after that for the next eight hours. A stationary phase measurement was also taken 25 hours after inoculation of the 24-well-plate.

During every measurement, a colony count and pH measurement of all four inoculated LB media samples was executed. The following day the CFU/ml could be determined and the collected data analysed as described in 3.6 Colony Counting.

3.10.b Growth Curve Experiment in FBS

The previously mentioned growth curve experiment was then repeated with FBS instead of LB media. FBS is used to model wound fluid and the measurements will be better comparable to the *in vivo* conditions of a chronic wound. A short overview of the experiment is illustrated in figure 24.

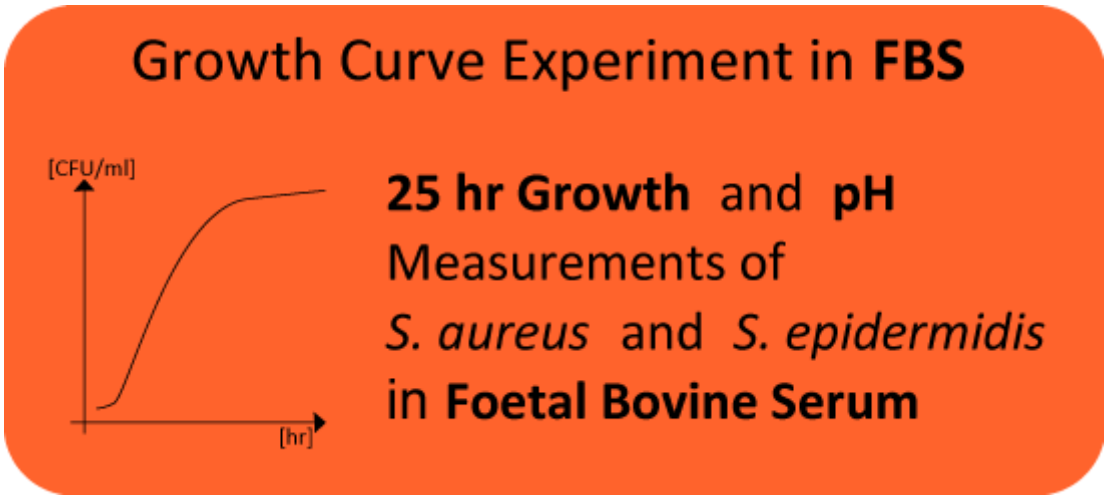


Figure 24: Growth Curve Experiment in FBS. This shows the measurements done in LB media over 25 hours. The expected outcome of the bacterial growth during that time is given on the left

The setup of the 24-well-plate was similar to the layout of the 3.10.a Growth Curve Experiment in LB media except FBS was used instead of LB media.

The first measurement was taken at inoculation time and every hour after that for eight hours and 25 hours after inoculation. One measurement consisted of a colony count for both bacteria solutions and their pH measurements. The CFU/ml could be calculated and analysed the following day as described in section 3.6 Colony Counting.

3.10.c Long Term Growth Curve Experiment in FBS

A growth curve experiment typically measured the bacteria growth and pH change of about eight hours and one stationary measurement after 25 hours. In order to see the big picture, however, a long term growth curve experiment has to be implemented. A general overview of the experiment is illustrated in figure 25.

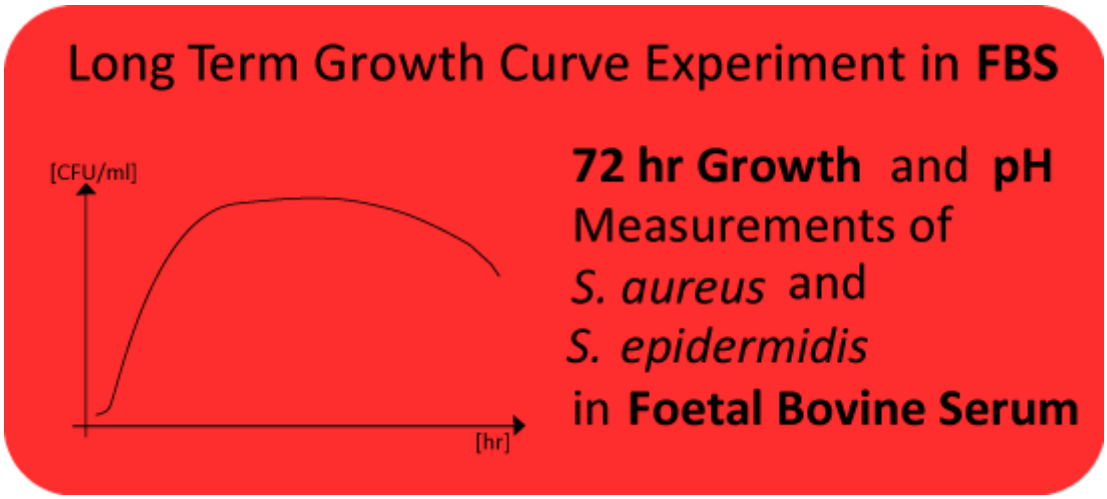


Figure 25: Growth Curve Experiment in FBS. This shows the measurements done in FBS media over 72 hours. The expected outcome of the bacterial growth during that time is given on the left.

For this measurement, overnight cultures of *S. aureus* and one of *S. epidermidis* were set up and two identical 24-well-plates were inoculated; one using *S. aureus* and one using *S. epidermidis*. The 24-well-plate layout was designed as follows. Four wells were filled with $1\ ml$ FBS each and inoculated with either *S. aureus* or *S. epidermidis*. Another four wells were also filled with FBS as a negative control to ensure the media is not contaminated. Furthermore, eight wells were filled with FBS coloured phenol red with four of them also inoculated with one of the two bacteria. These wells are being set up as a control to the pH measurements.

The first measurement was taken at t=0 hours and then twice a day after inoculation for a total of 72 hours. At every measurement, a colony count and pH measurement was carried out. The colony counting protocol is described in section 3.6 Colony Counting.

4. Results

Firstly, the results of the bacterial characterisation are presented in order to get familiar with the growth and handling of the bacteria. Secondly, the pH sensor validation results for the use in LB media are described. Thirdly, the results for the growth curve experiment with corresponding pH measurement in LB media and FBS over 25 hours are illustrated. And finally, the results of a long term growth experiment in FBS over 72 hours are described to be able to see the big picture.

4.1 Bacteria Characterisation

Colony morphology and general characteristics of *S. aureus and S. epidermidis* were explored to determine relevant growth features and factors.

The results of a colony count for both bacteria strains in LB media overnight in either a stationary 24 well plate show that *S. aureus* seems to grow faster than *S. epidermidis*. The final colony count of the broth the following day yields the total numbers of 5.81×10^8 CFU/ml with a SE of 2.80×10^8 CFU/ml for *S. aureus* and 2.41×10^8 CFU/ml with a SE of 1.45×10^6 CFU/ml for *S. epidermidis*. Both are expected to have reached the stationary phase. Therefore, the maximal dilution factor for the following experiments is chosen to not exceed 10^{-7}.

In a second characterisation experiment both bacteria were grown on MSA overnight. Figure 26 shows the MSA plate of *S. aureus* the following day compared to a plain MSA plate. The colour changed from light red to yellow which is due to the phenol red content in the MSA. This means that the pH dropped below 6.8 where the colour is yellow. This is happening

because *S. aureus* is able to ferment mannitol as its carbon source and produce organic acids as end products [Mahon et al. (2015)].

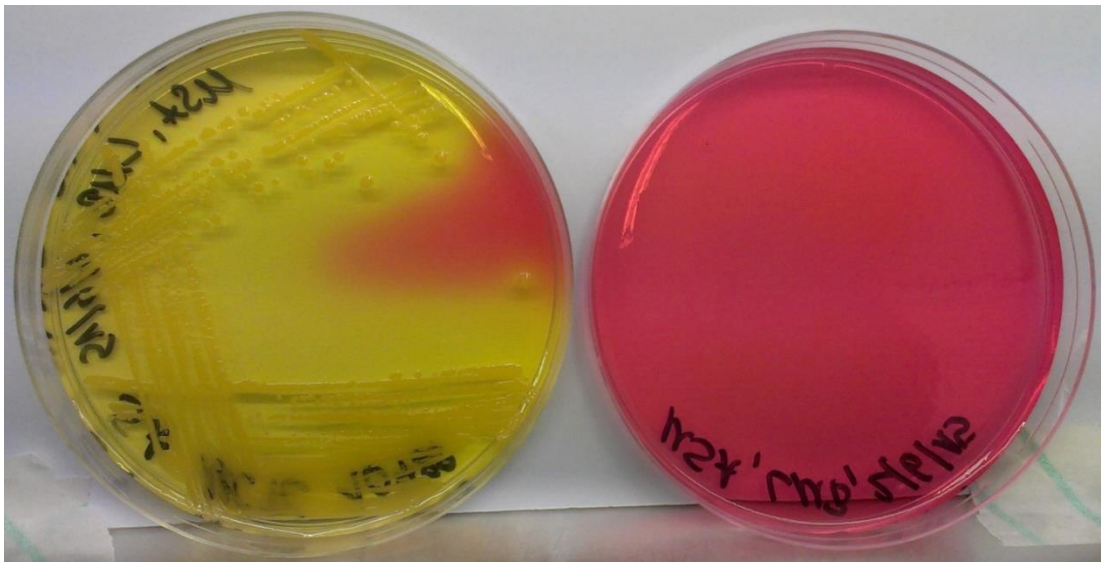


Figure 26: Overnight growth of S. aureus on MSA. Note the yellow colour change on the left plate with S. aureus compared to the sterile MSA plate on the right that was also left in the incubator overnight.

Figure 27 shows the growth of *S. epidermidis* on MSA after overnight incubation. Close inspection of the colonies reveals that *S. epidermidis* appears to grow more slowly than *S. aureus*, confirming the results of the previous characterisation experiment. Furthermore, the colonies were smaller and the colour of the MSA changes from slight red to pink, indicating an alkaline pH. This means that *S. epidermidis* is not able to ferment mannitol into organic acids and most likely alkaline substances like ammonia are produced instead.

S. aureus is able to ferment mannitol as its carbon source while *S. epidermidis* cannot [Murray et al. (2009)]. The mannitol fermentation produces organic acids which lower the pH. Since *S. epidermidis* cannot break down mannitol, the pH does not decrease, but it increases due to basic metabolism and the thereby produced end products.

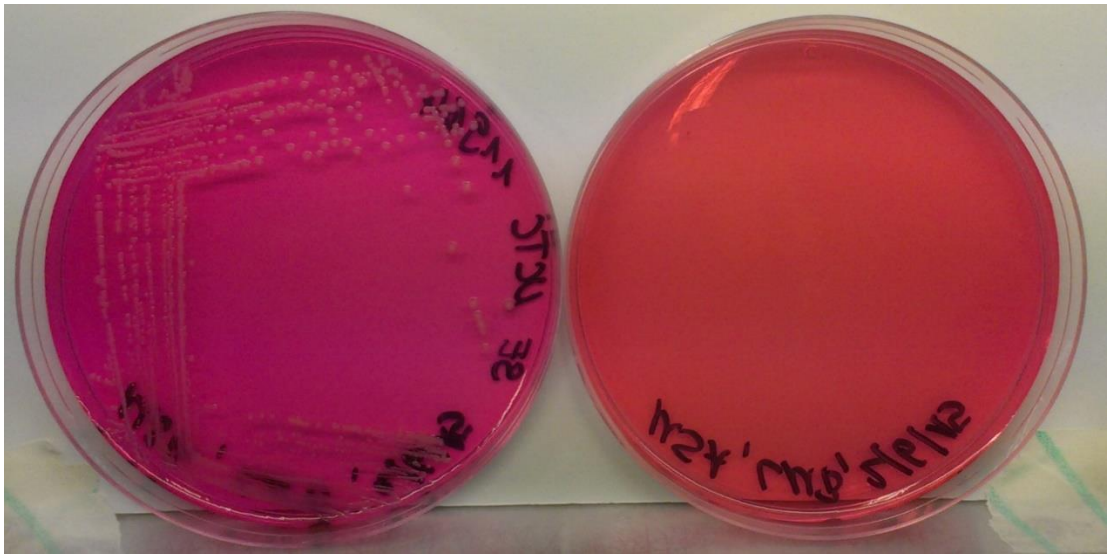


Figure 27: Overnight incubation of S. epidermidis on MSA. MSA with S. epidermidis is shown on the left. It shows a slightly pink colouring compared to the sterile MSA plate.

4.2 pH Sensor Measurement Verification of LB Media

To verify that the pH measurement was not affected by the LB media ingredients, an experiment was implemented. Firstly, the working electrode was measured with a glass electrode as reference for three different concentrations of tryptone (0.05 g/ml, 1 g/ml, 2 g/ml), yeast extract (0.25 g/ml, 0.5 g/ml, 0.1 g/ml), sterile LB medium and pH4 and pH 7 calibrations solutions. Secondly, the printed Ag/AgCl electrode was used with a glass electrode as a reference to it for the same solutions. The results of the printed working electrode showed a different potential for the different solutions. In general the measurements were stable. After a time period of 60s, the value of the recorded potential was stabilized, for example the potential of tryptone was 15 mV while the potential for the chloride adjusted tryptone solution was 8 mV.

The more important second test was to identify if the reference electrode measurement was affected by the ingredients themselves. In figure 28, the reference electrode measurements

are presented. The black bars show the LB media ingredients in distilled water for their three concentrations. A change in concentration recognisably lead to a difference in the measured potential of the reference electrode. Once the solutions were chloride adjusted, however, the measurements were fairly stable at approximately 80 mV. This can also be seen from the red bars in figure 28. This stable potential was most likely due to the chloride which was dominating the voltage response of the sensor. Another measurement of the calibration solutions and the LB media showed that their measured voltage levels were at a similar potential level.

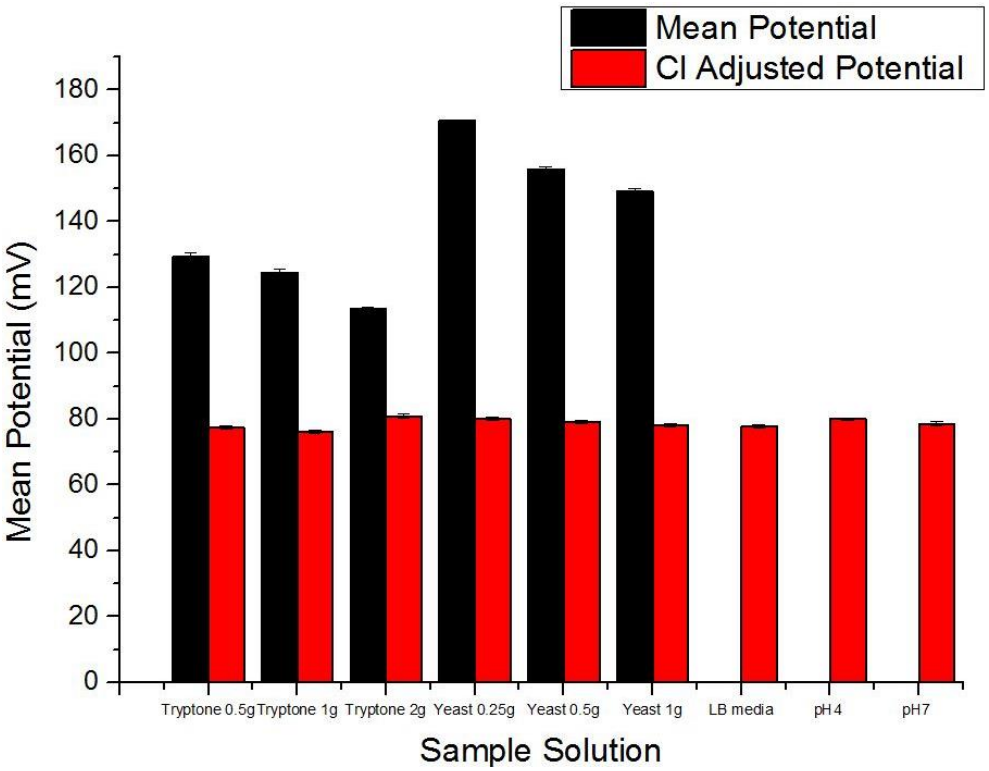


Figure 28: Reference electrode behaviour for LB media components in different Concentrations. This shows the measurements of the printed reference electrode of the sensor for the different concentrations of the LB media ingredients; tryptone (0.5g, 1g and 2g per 100ml) and yeast extract (0.25, 0.5 and 1g per 100ml). The black measurements show the ingredients in distilled water while the red bars show the solutions after they were chloride adjusted. The chloride seem to have dominated the response of the sensor measurement. Furthermore, the calibration solution measurements and the LB media itself were tested with this setup (n=6, error bars represent ±SE).

These results show that the disposable sensor was compatible with LB media and the calibration solutions did not have to be adjusted further to be used with this setup.

It should, however, be noted that the electrodes should at no point get in contact with water at uninsulated areas of the sensor. The measurements were not stable and varied with a range of 50 mV even after 60 seconds. If this happens at any point during an experiment, a new, dry sensor has to be swapped with the wet one and the measurements have to be repeated.

The sensors produced for this thesis all had similar measured calibration voltages. The mean of the measured pH 4 Voltages was V_{pH4} = 0.97 V (± 0.003 SE) and of pH 7 was V_{pH7} = 1.27 V (± 0.004 SE) all derived from ten separate pH calibrations of the sensors.

4.3 pH Measurements in Bacterial Cultures

In the following section, the results of the experiments investigating the pH and bacterial growth correlation are presented. At first both bacteria were grown in LB media for 25 hours with an hourly measurement for the first 9 hours. Afterwards the same experiment was repeated in FBS followed by a long term growth experiment over 72 hours in FBS.

4.3.a Growth Experiment in LB Media

A growth curve and the corresponding pH values of the media were determined for *S. aureus* and *S. epidermidis* over 25 hours. LB media was inoculated with overnight cultures (about 15 hr) of the two bacteria and hourly measurements were taken for the first eight hours of the experiment followed by a measurement in stationary phase after 25 hours. The measurements for *S. aureus* are shown in figure 29 and the ones for *S. epidermidis* are shown

in figure 30. A negative control in form of sterile LB media was included in the experiments. The sterile LB media was stable for the entire duration of the measurement. There was one outlaw measurement of sterile LB two hours after start of the experiment where the pH value was as high as pH of 7.47 while the rest of the pH measurements were in a range of 6.88 and 7.07. The outliner measurement is most likely caused by differences in the sensor production or measurement uncertainties.

S. aureus was in lag phase for the first hour of the experiment. The following log phase continues until 25 hours after experiment's start. In the beginning of the log phase, the corresponding pH dropped rapidly until it reached an acidic plateau at pH 6.39 (±0.012 SE) after 4 hours following inoculation of the media. This stands in clear contrast to the fairly stable pH of the LB media itself. At the end of the log phase, before the bacteria growth reached stationary phase the pH rose continuously until it reached a neutral pH of 7.04 (±0.012 SE) after 7 hours. The pH continued to rise continuously for the rest of the experiment and reached its maximum for this experiment at 7.96 (±0.034 SE) after 25 hours.

S. epidermidis generally followed the same trend. However the pH change was less incisive. The lag phase held on for the first two hours of the experiment until the CFU/ml increased continuously in the log phase. During the log phase the pH of the solution also dropped to slightly acidic levels. An acidic level plateau of pH 6.66 (±0.124 SE) forms after three hours which is different from the pH of sterile LB media which stayed stable. The pH of the inoculated media started rising at the end of the log phase and continuously rose in the stationary phase. After 25 hours the maximum pH of this experiment was reached with 7.68 (±0.066 SE).

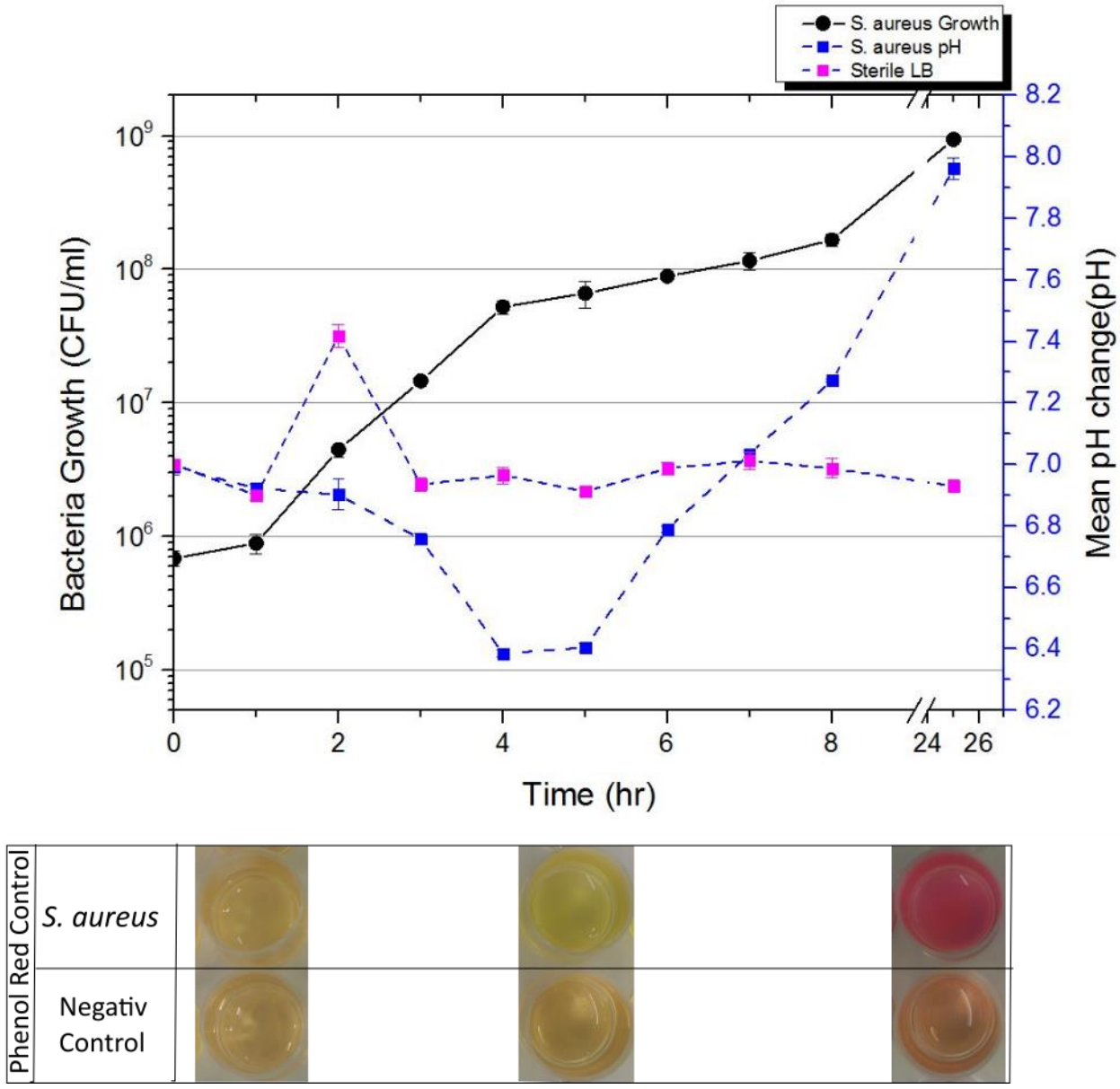


Figure 29: Growth curve experiment in LB media with S. aureus. The mean growth is plotted in black corresponding to the left y-axis while the pH is plotted in blue with dashed lines corresponding to the right y-axis. Below the graph the estimated growth phase of the bacteria is labelled and a quantitative picture of the phenol red control is added for several time steps (n=4, error bars represent ±SE).

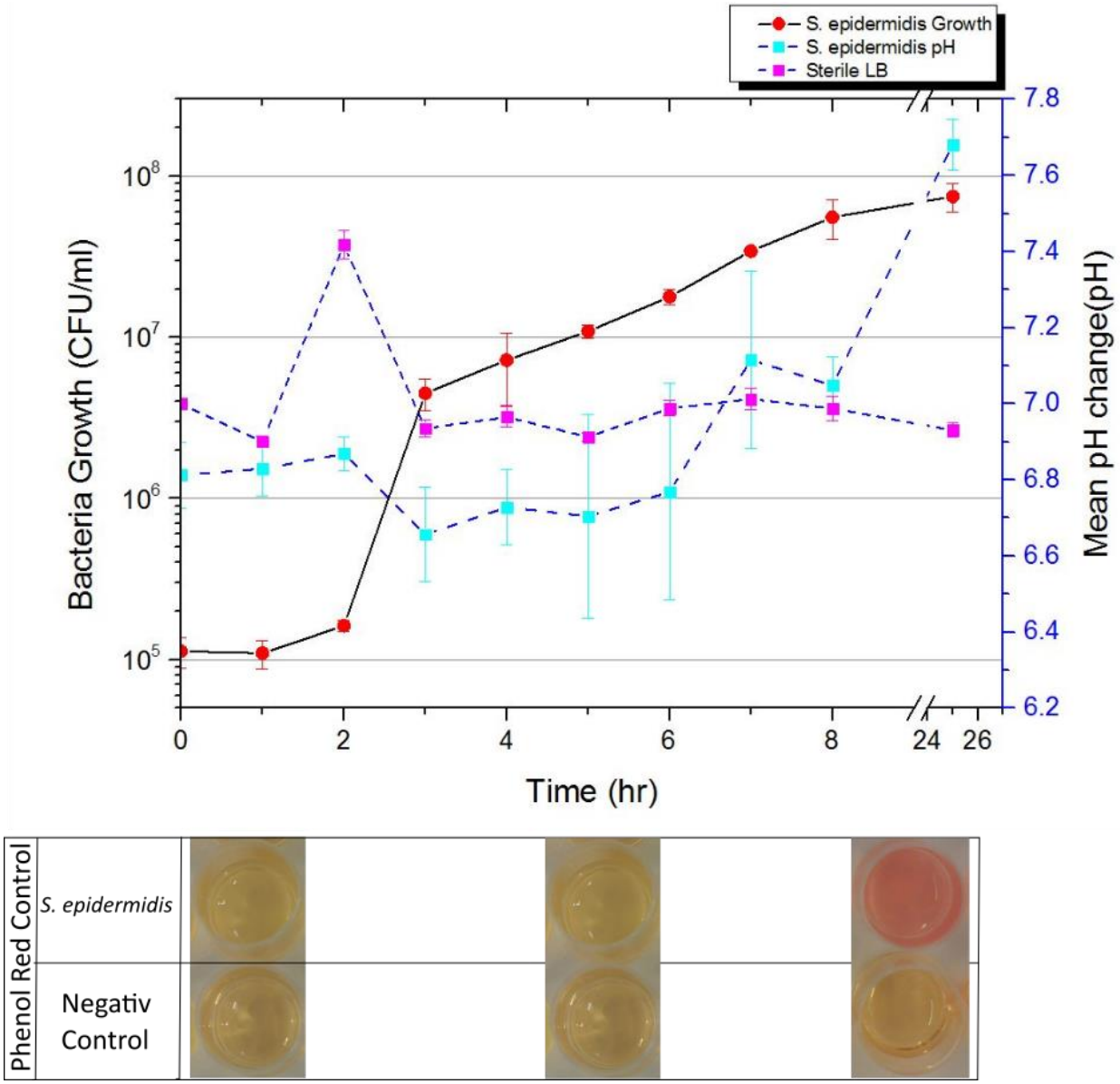


Figure 30: Growth curve experiment in LB with S. epidermidis. The mean growth is plotted in black with red circles corresponding to the left y-axis while the pH is plotted in a blue line with light blue squares corresponding to the right y-axis. Below the graph the estimated growth phase of the bacteria is labelled and a quantitative picture of the phenol red control is added for several time steps (n=4, error bars represent ±SE).

4.3.b Growth Curve Experiment in FBS

To evaluate the pH change of infected wound fluid in a better *in vitro* model, the 25 hour growth curve experiment was repeated in FBS. In figure 31 the mean values of pH and estimated bacteria numbers of *S. aureus* are plotted. Furthermore, the pH measurements of sterile FBS were added to the graph as a reference.

The experiment started in the log phase. The immediate growth of the bacteria is possibly explained by the good growth environment with preheated media and the fact that the bacteria used to inoculate the culture were already in their growth phase. The bacterial number was growing continuously until it reached the stationary phase after approximately 20 hours.

The pH of the sterile FBS was reasonably stable around a value of 8.38. In the end of the log and beginning of the stationary phase the pH value rose slowly to a maximum of 8.74 (±0.018 SE). Compared to the pH change of the media containing *S. aureus,* these changes were minimal. During the log phase the pH decreased significantly from a normal FBS pH of 8.36 (±0.058 SE) at t=1 hours, to a minimum of 6.33 (±0.02 SE) at t=7 hours. Afterwards the pH was increasing rapidly. The final pH value was 8.10 (±0.04 SE). The pH drop of about 2 pH levels was very significant and the pH profile created by the decrease and following increase in pH seems unique. The maximum difference between the pH of sterile FBS and inoculated FBS is 1.97.

The growth of *S. aureus* in LB media was similar to the growth in FBS. While the growth curve experiment in LB media started in the lag phase, the FBs experiment started in the log phase already. This is mostly due to the phase the bacteria were in at inoculation of the media. Both

bacteria numbers increase logarithmically until they reach their maximum number after 25 hours. The final bacteria counts for LB media were 9.4×10^8 CFU/ml ($\pm 5.32 \times 10^7$ SE), and therefore slightly higher than the bacteria numbers in FBS with 2.48×10^8 CFU/ml ($\pm 4.84 \times 10^7$ SE).

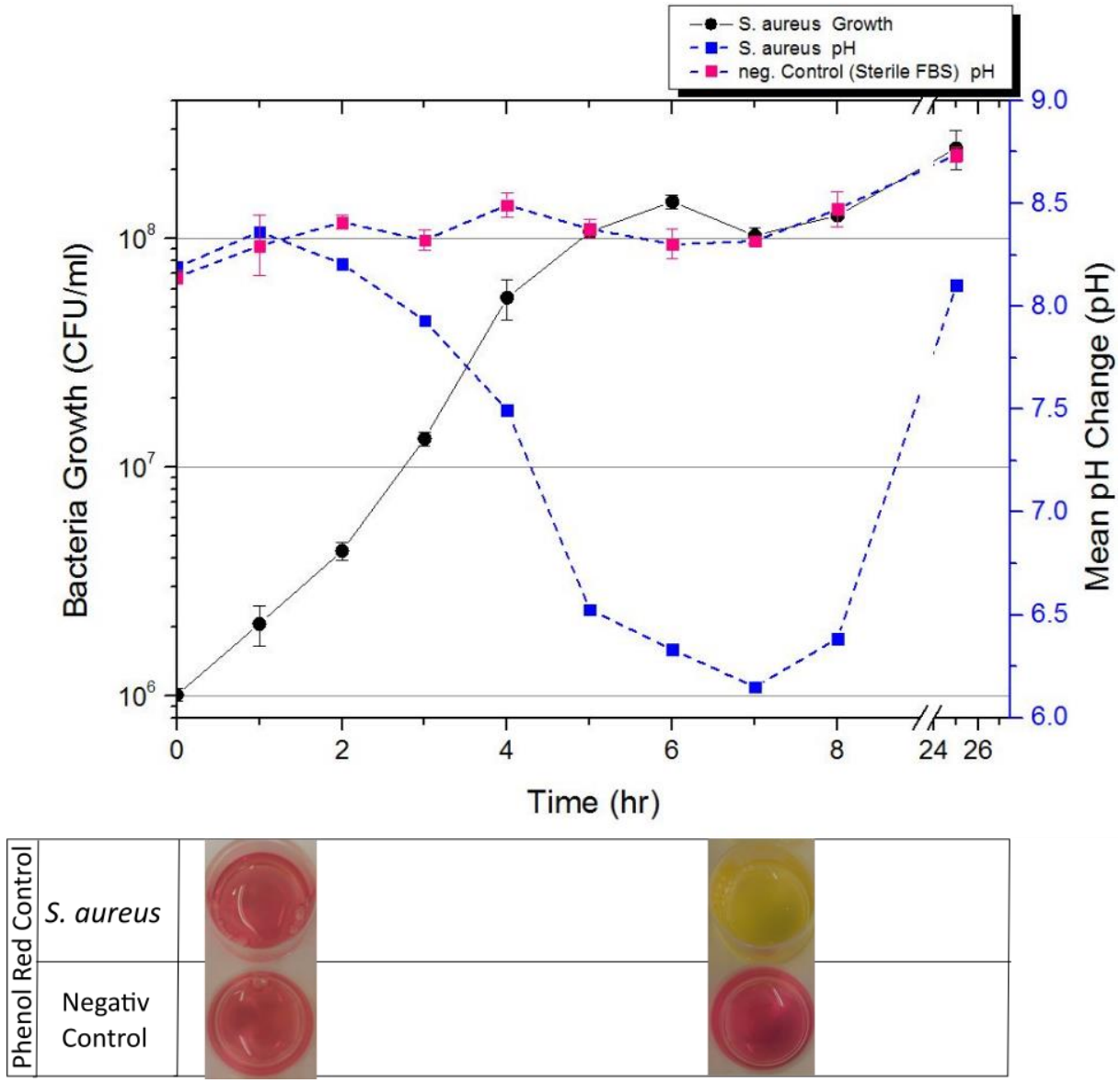


Figure 31: Growth Curve Experiment in FBS with S. aureus. The bacterial growth (black) and pH change (blue) of S. aureus in FBS over 25 hours is measured and plotted here. As a reference the pH change of sterile FBS (pink) was also included in this graph. Below the graph the estimated growth phase of the bacteria is labelled and a quantitative picture of the phenol red control is added for several time steps (n=4, error bars represent ±SE).

In figure 32 the pH of sterile media and inoculated FBS with *S. epidermidis* is plotted on the right y-axis while the left y-axis shows the corresponding bacteria growth.

The stationary phase lasts for approximately the first two hours of the measurement until the log phase of the bacterial growth sets in.

The pH of the media inoculated with *S. epidermidis* follows the pH values of sterile FBS for the first three hours. During the log phase the pH of the inoculated media decreases slowly. The biggest difference between the two pH values is 0.58 and it is reached after 25 hours.

The bacterial growth of *S. epidermidis* during the growth curve experiment in LB media and FBS appears to be shifted. While the log phase in LB media started approximately two hours after the experiment's start, the log phase's start in FBS is hard to grasp and seems to be shifted and started only four to six hours after experiment start. The final bacterial numbers after 25 hours were, however, very similar. For LB media the final bacterial numbers were 7.5×10^7 CFU/ml ($\pm 1.52 \times 10^7$ SE) and in FBS the bacterial count was 4.78×10^8 CFU/ml ($\pm 4.21 \times 10^7$ SE). These results are however, not yet sufficiently proven and more studies in this area have to be conducted.

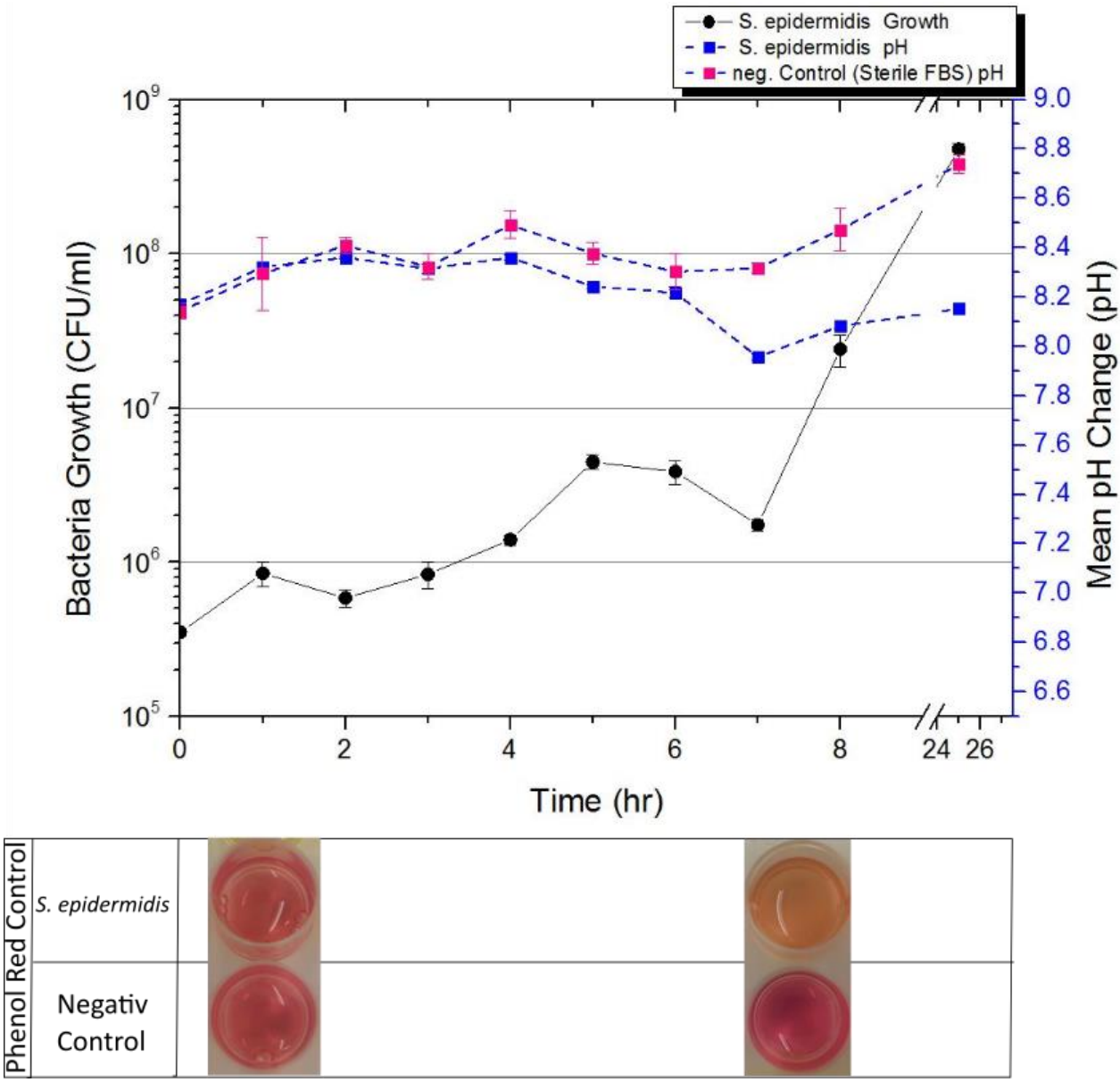


Figure 32: Growth curve experiment in FBS with S. epidermidis. The bacterial growth (black) and pH change (blue) of S. epidermidis in FBS over 25 hours is measured and plotted here. As a reference the pH change of sterile FBS (pink) was also included in this graph. Below the graph the estimated growth phase of the bacteria is labelled and a quantitative picture of the phenol red control is added for several time steps (n=4, error bars represent ±SE).

4.3.c Long Term Growth Experiment in FBS

To be able to investigate the entire pH change of the inoculated media, a long term growth experiment was conducted. With the help of this data, the long term pH profile of specific bacteria strains was intended to be identified.

Over 72 hours, the growth of *S. aureus* and *S. epidermidis* was monitored. Additionally, the pH change of FBS was determined. The first measurement of FBS was approximately one pH level below the rest of the measurements. This can also be seen in the slight colour change in the phenol red control below figures 33 and 34. No coherent explanation for this occurrence could be found, but it is possibly due to an oxidation or another reaction of FBS after it was defrosted and exposed to oxygen rich environment. All following pH values are stable in a range of 8.61 to 8.9.

The recorded data for *S. aureus* are shown in figure 33. The measurement began in the log phase which lasted until approximately 20 hours after the experiment's start. After that it reached the stationary phase and the bacterial numbers declined during the death phase which occurred after around 55 hours. The pH for *S. aureus* decreased rapidly to an acidic pH of 6.23 (±0.43 SE) only eight hours after inoculation of the FBS in the middle of the log phase. Afterwards the pH rose continuously to a maximal alkaline pH of 8.95 (SE ±0.48) after 72 hours of the experiment. The rate of increasing pH appeared to increase at the end of the log phase and during the death phase while it slowed down during the stationary phase.

Figure 34 shows the bacteria growth and pH change of FBS inoculated with *S. epidermidis*. The experiment also started in the log phase and reached the stationary phase approximately 15 hours after inoculation. The bacteria viability of *S. epidermidis* declined more drastically than in the experiment with *S. aureus*. About 40 hours after the experiment's start the death

phase occurred and the number of viable bacteria dropped almost two entire decades compared to the bacteria count of the stationary phase.

The pH of the *S. epidermidis* solution already started at a slightly alkaline pH of 7.77 (SE ±0.015) and stayed mostly alkaline in a range of 7.53 to 8.55. The general trend of the *S. epidermidis* pH was towards alkaline and it was mostly stable. Generally the pH change of *S. aureus* was more drastic than the change of the wells inoculated with *S. epidermidis*. Furthermore, it was noted that the pH profile of both bacteria were clearly different.

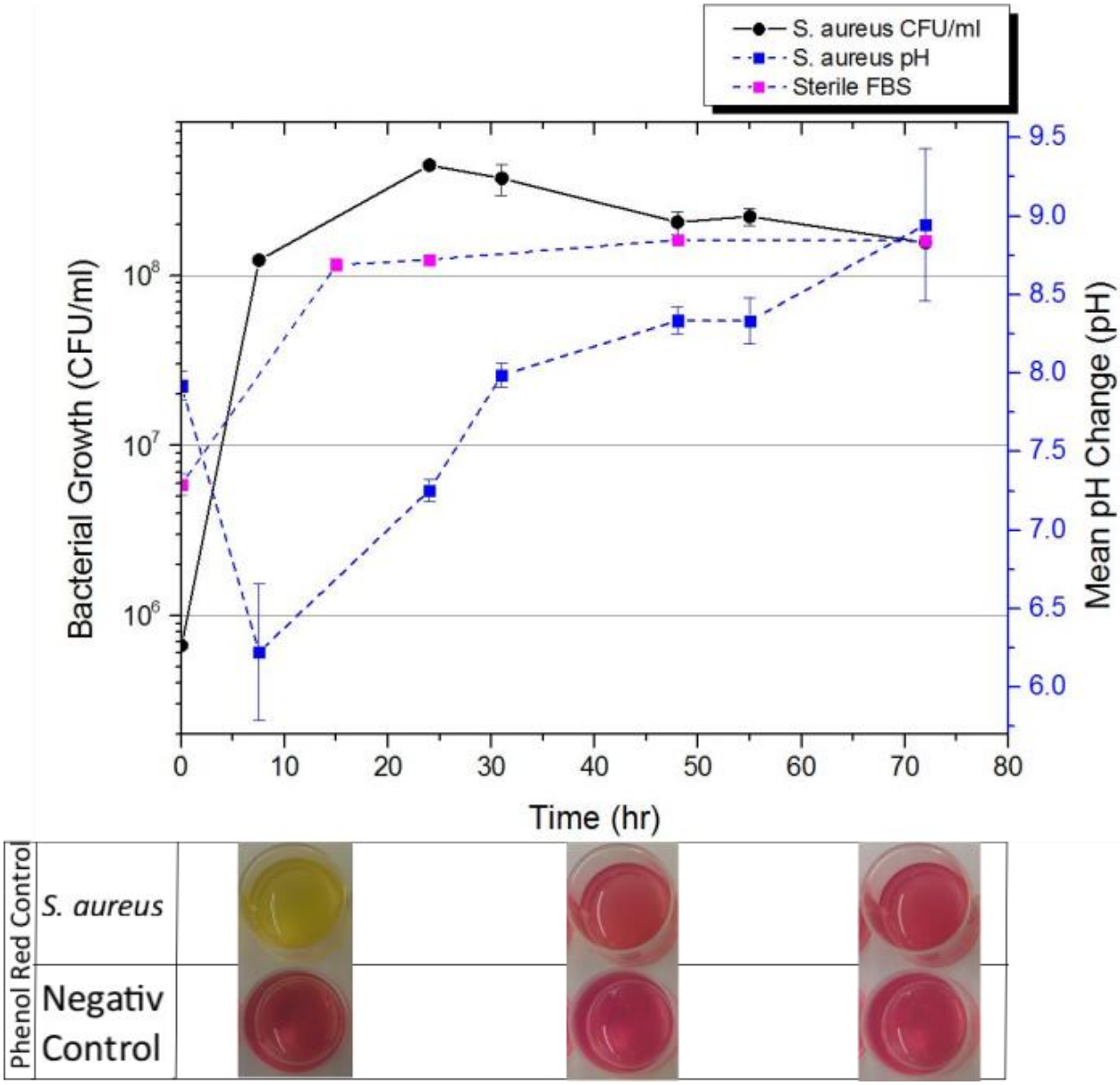


Figure 33: Long term growth curve experiment with S. aureus. S. aureus was grown in FBS for 72 hours. One count and pH measurement was done at inoculation t=0, and every 8 and 24 hours after that. This figure shows the plotted data of the bacterial count on the y-axis of the left in black and the measured pH values in blue corresponding to the y-axis on the right. Below the graph the estimated growth phase of the bacteria is labelled and a quantitative picture of the phenol red control is added for several time steps (n=4, error bars represent ±SE).

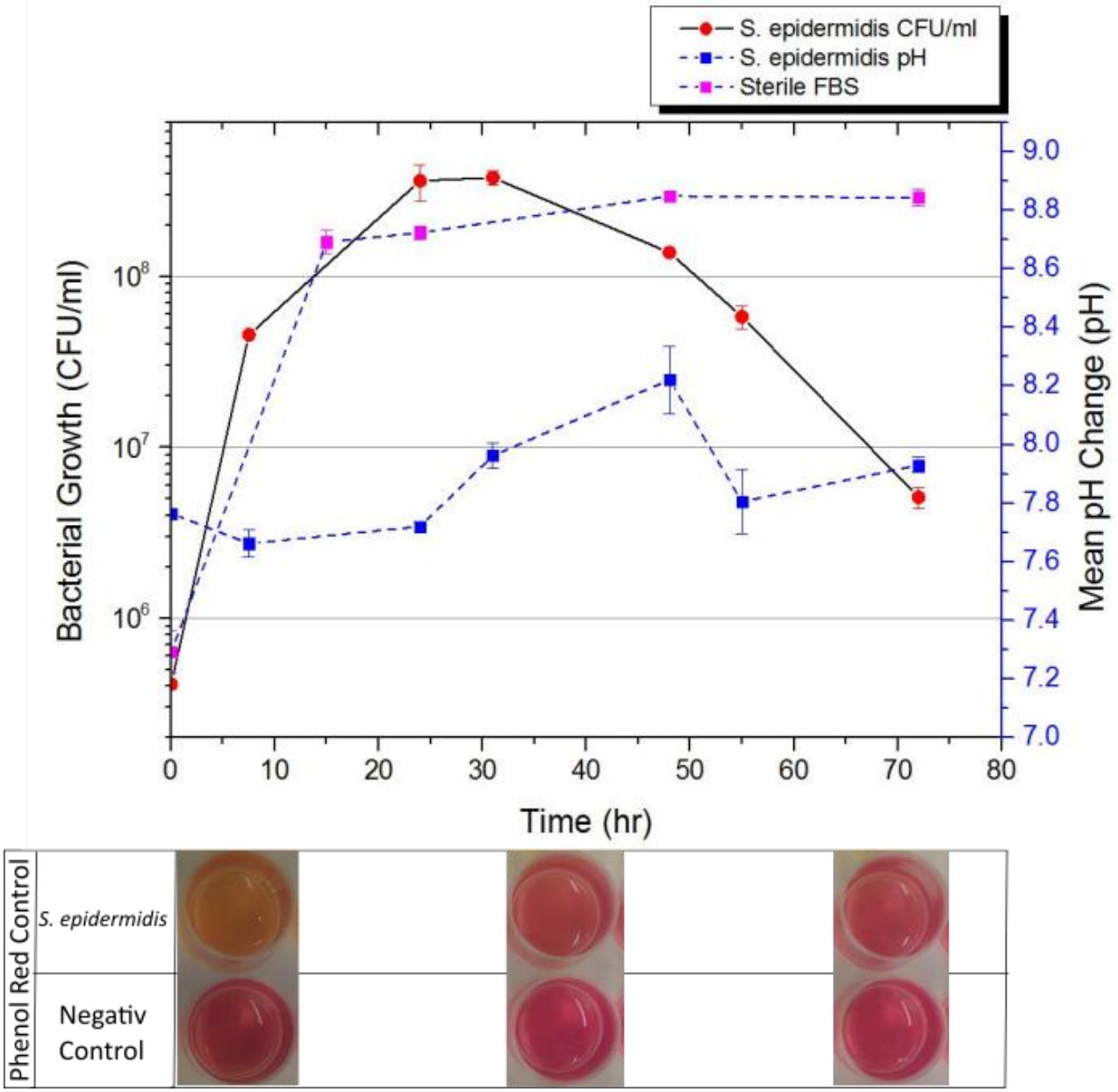


Figure 34: Long term growth experiment with S. epidermidis. S. epidermidis was grown in FBS for 72 hours. One count and pH measurement was done at inoculation t=0, and every 8 and 24 hours after that. This figure shows the plotted data of the bacterial count on the y-axis of the left with a black line and red circle and the measured pH values in a blue line with light blue squares corresponding to the y-axis on the right. Below the graph the estimated growth phase of the bacteria is labelled and a quantitative picture of the phenol red control is added for several time steps (n=4, error bars represent ±SE).

In summary, it can be said that a clear difference between the pH changing profiles correlating with the bacterial growth of *S. aureus* and *S. epidermidis* were noted. For *S. aureus* a rapid pH drop of two pH levels, followed by a pH rise to alkaline levels could be identified as the typical pH profile of the bacteria. In contrast, the pH profile of *S. epidermidis* was less obvious but follows a similar trend. The pH first also drops approximately 0.2 pH levels followed by a rise in pH by about 0.8 pH values. Both pH profiles are differentiable which allows bacterial infection detection and possibly strain differentiation in chronic wounds.

5. Discussion

In this feasibility study, the correlation between pH change and bacterial presence in simulated wound fluid is investigated. If a link between the two can be established, chronic wound infections can be detected early and will not further delay wound healing. Furthermore, bacterial strains could possibly be differentiated between each other by comparing known pH profiles helping to choose appropriate antibiotics and treatment possibilities. Patients discomfort and antibiotic misuse could be minimised and chronic wound healing accelerated.

The bacteria and sensor characterisation experiments showed that the serial dilutions for the measurements will never exceed 10^{-7} and the disposable pH sensor is compatible with LB media. The device was validated and can yield plausible results.

From previous studies it is known that the disposable pH sensor used in this thesis is working accurately with physiological samples and foetal bovine serum [Milne et al. (2014)]. The accurate working function in LB media was established in this thesis and the sensor was used for all measurements.

A general pH profile for *S. aureus* and *S. epidermidis* has be identified during the experiments conducted in this study. The pH trend of *S. aureus* is shown in figure 35. At inoculation with the bacteria, the measured pH was similar to the media pH at around neural pH level. During the log phase of *S. aureus* the pH dropped continuously about two pH levels to acidic values. Afterwards the pH started to increase and changed from acidic over neutral to alkaline pH values. The final pH value of the LB media measurement and the long term growth

experiment in FBS showed a pH level of one above the media pH. This pH profile is significant and follows the same trend in both, LB media and FBS.

For *S. epidermidis* the pH change was less significant and stayed fairly stable. In LB media the pH firstly dropped only 0.2 pH levels below the media pH during the log phase and increased about 0.6 pH levels above the media pH when it settled in stationary phase. In FBS the recorded pH value for *S. epidermidis* stayed below the pH of sterile FBS for the entire duration of the experiment. A drop from a neutral level to slight acidic levels is visible, followed by a rise in pH during the stationary phase. Once *S. epidermidis* enters death phase, however, the pH started dropping again.

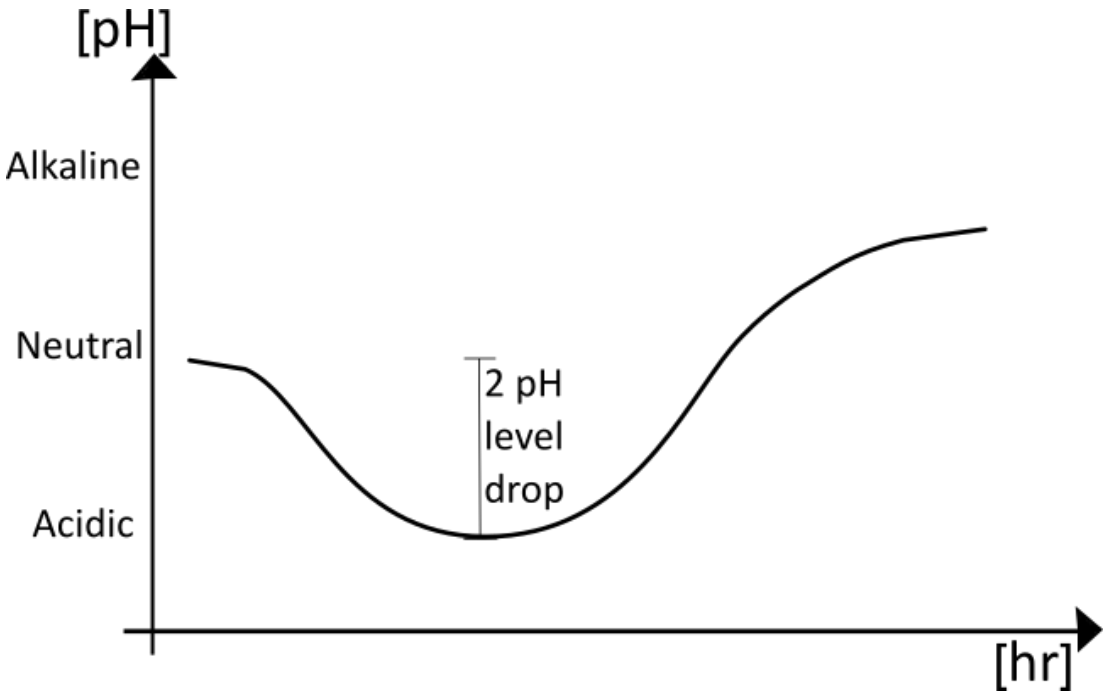


Figure 35: General pH profile of S. aureus during growth. The pH of the beginning of the experiment is as neutral as the media used. During the log phase of S. aureus, the pH level drops drastically up to two entire pH levels. After the pH dropped to acidic levels, the pH begins to rise again and continues rising up to alkaline pH values.

In the measurements with both bacteria the difference between the pH change in LB media and FBS is visible. This could be due to the fact that LB media is buffered, which is usually the case for any growth medium. This means that the media pH is regulated and will stay fairly stable around a chosen pH [Mahon et al. (2015)]. It is therefore especially interesting that a pH change in a range of 6.4 and 8.0 is detectable during the experiment. A pH drop of 2 pH levels like in FBS for *S. aureus* is not to be expected in LB media. FBS, like other serums uses protein buffers to control its pH. However, the FBS used in this study was heat inactivated. This is the reason such a big pH change could occur during the measurements.

The pH profile of *S. aureus* during these experiments is unique and can be easily differentiated from the pH profile of the negative control or of *S. epidermidis*. It may be possible to differentiate different bacteria species by comparing the collected data to known pH profiles. This is, however, not sufficiently proven and has to be further investigated.

Both bacteria profiles, however, also show similarities. For example, a pH drop followed by a rise in pH can be detected in both bacteria even though the magnitude of the drop and rise is different for both bacteria. It is, however, plausible to assume a similar behaviour in all bacterial infections in chronic wounds. Recent studies showed that infected wounds generally have a high pH which would support this claim [2, Uzun et al. (2011)].

The underlying mechanisms of the pH profile are not yet fully understood and more research has to be conducted in that field. However, a few speculations were made, collected and are presented in the following paragraphs.

The pH drop into acidic levels can possibly be explained by the bacteria metabolism and the thereby produced acidic end products. In section 2.2.f Metabolism other metabolic pathways are presented.

The pH drop into acidic levels can possibly be explained by the bacteria metabolism and the thereby produced acidic end products. In section 2.2.f Metabolism other metabolic pathways are presented. The pH drop could also occur from increased proton efflux out of the bacteria.

The following pH rise of the bacteria is more difficult to explain through traditional metabolic pathways, and could be caused by other bacterial mechanisms. A possible explanation is that the bacteria gene expression is altered due to exposure to an acidic environment. The so-called Mild Acid Stimulation (MAS), further explained in 2.2.f Metabolism, is thought to cause a gene expression alteration in order for the bacteria to adapt to the new environment. A study from Weinrick et al. (2004) found that *S. aureus* produced acidic end products which lowered the media pH two entire pH levels when the gene expression of *S. aureus* adapted to the acidic pH via MAS and altered its gene expression accordingly. The study further suggests that a pH change similar to the one found is likely to be a defence mechanism. Only bacteria able to adapt to such big pH changes can be found in the infected wounds, while bacteria incapable of this response will be terminated [Weinrick et al. (2004)].

A more drastic way of gene expression alteration is the Acid Tolerance Response (ATR) [Rode et al. (2010)]. Bacteria might try to regulate their cytoplasmic pH and adapt their metabolism accordingly. This might not only change the pH of the cytoplasm, but also the environmental pH in the media. Furthermore, during the stationary or decline phase, bacteria lysis will spill cytoplasm in the media which might further alter the pH. These are, however, only

speculations and further studies will have to investigate this behaviour in order to draw sound conclusions.

It has to be kept in mind that all findings in this theses were discovered *in vitro* with FBS as chronic wound fluid model and are only partly applicable to real *in vivo* environment of chronic ulcers. An *in vivo* situation is always more complex due to several reactions and cofactors within the wound area that cannot be simulated in a simplified *in vitro* experiment. One major point is the presence of far more proteins and microorganisms in the wound site and blood. The FBS used was heat treated through which many microorganisms were inactivated and proteins were denatured. Furthermore, it has to be noted that the protein presence in chronic wounds is decreased compared to normally healing wounds, but once the wound started healing the protein amount present in the wound fluid increases which is not simulated by FBS [Gethin et al. (2008)]. A chronic wound is colonized by several bacteria species and the wound fluid is filled of enzymes like proteases and other proteins including various growth factors [Wysocki et al. (2012), Wolcott et al. (2009), Shai et al. (2005) and Schneider et al. (2007)]. The proteins present in chronic wounds can also serve as pH buffers for the wound fluid, making it more difficult for the produced substances to change the pH within the wound site.

The pH measurements change with temperature [Puchberger-Enengl et al. (2012)]. This is why the temperature of the wound will have to be assessed as a second parameter if the wearable pH sensor is integrated in wound dressings. A typical infection sign is a rise in temperature at the wound site [Puchberger-Enengl et al. (2012)]. This would however, change the measured pH of the wound and would further influence the conclusions about

the measurements even though any change can be compensated with an equation. A multi parameter sensor including pH, temperature and other relevant parameters, for example moisture, is a promising technique for sufficient, continuous wound assessment without additional stress for the wound site in form of wound dressing removal.

More research will have to be done to determine if a chronic wound environment is aerobic or anaerobic. Depending on this the metabolism and end products of the bacteria will vary and the measured pH could be invalid with the current technique. The Bohr-effect that was mentioned in 2.2.f Metabolism helps to supply a healing wound with more oxygen due to its low pH. If a wound is, however, infected or is already chronic, the oxygen supply will be limited. Consistent conditions and wound dressings should be used for further *in vivo* studies.

Another big difference between the *in vitro* and *in vivo* situation is the variation of wound pH and bacterial growth due to wound dressings. Wound dressings have two ways of influencing the pH change in the wound; actively and passively. The choice of wound dressings is wide and ranges from acidic over impermeable to silver dressings. While acidic wound dressings influence the wound fluid pH actively, silver and impermeable bandages manipulate the pH only passively.

Acidic wound dressings are designed to actively lower the wound pH and try to promote wound healing that way [Schneider et al. (2007), Gethin et al. (2008)]. This will interfere with the original pH change in the wound fluid and might dominate the pH profile of the wound from the point of application. It has to be tested if the pH change profile of bacterial infection is detectable under these conditions or if it is impossible to combine the two principles.

Silver dressings have been shown to inhibit bacterial growth in the wound [Percival et al. (2014), Uzun et al. (2011)]. However, most modern wound dressings have one or another antimicrobial substance in them to avoid microbial growth in wounds [Uzun et al. (2011)]. Minimizing the amount of bacteria in the wound site translates to fewer end products produced during the metabolism which would have otherwise change the pH. Furthermore, it should be noted that the silver dressings are more effective at acidic pH values of 5.5 rather than at neutral pH of 7 or alkaline pH values [Uzun et al. (2011)].

Wounds where non-permeable wound dressings were used have a more acidic milieu than wound with permeable dressings [Schneider et al. (2007)]. This situation touches the previously discussed anaerobic or micro aerophilic environment. A bacteria's metabolism changes from the more energy efficient respiration to fermentation in anaerobic environment. In 2.2.g Metabolism the possible fermentation pathways were shown and it can be seen that most of them produce acidic end products which would explain the acidification of the wound for non-permeable wound dressings.

A modern technique of chronic wound treatment is vacuum dressing [Pruksapong (2011)]. It is nowadays mostly practiced on in-bed patients but is currently implemented in an efficient portable vacuum dressing for out-patients [Pruksapong (2011)]. The metabolism of the bacteria is altered in vacuum and further research of bacterial pH profiles has to be conducted in vacuum to decide if the pH profile of the wound can still be linked to bacterial contamination and, therefore, both techniques are compatible.

6. Future Directions

More research will have to be done with several different focus points to verify the insights that were found in this study and deepen the knowledge about it.

Different bacteria strains should be tested in growth curve experiments to identify their pH profile and verify the assumption that bacteria presence will increase the pH of FBS and chronic wounds. An interesting bacteria to investigate would be typical pathogens of chronic wound infections including P. aeruginosa. Once more bacteria would have been tested, their pH profile could be compared to each other and it can be evaluated if the pH profile yields enough information to differentiate between different bacterial species.

The effect of bacterial colonisation and multiple bacterial strains present in the same wound gives rise to the question whether the bacteria inhibit each other and control their growth rates during growth phase as they do when they are in equilibrium on the skin surface.

The pH profile during healing and the question why chronic wounds develop should also be investigated further in order to draw decisive conclusions relating to pH change in wounds.

As already mentioned in 5. Discussion, the pH profile measurements should be carried out *in vivo* in order to verify the results gained in this *in vitro* study. Furthermore, it should be investigated whether the wound environment is aerobic or anaerobic and what effect different wound dressings have. The use of different wound dressings has the potential to disrupt the natural pH change with infection and alter the bacterial response during growth.

Finally, it would be interesting to investigate if the infection detection technique according to the pH profile of the wound is applicable to other wounds, for example normal healing

wounds or burn wounds. This would broaden the scope of this technique and would be beneficial in all applicable fields to detect bacterial infection as early as possible.

7. Conclusion

In this study the correlation between bacteria presence and pH was investigated. The necessary knowledge about normal wound healing, chronic wound, normal pH change in wounds and bacterial behaviour, growth and metabolism was presented for better understanding of the discussion of the results. Growth curve experiments of *S. aureus* and *S. epidermidis* were implemented in LB media and FBS over 25 hours. Moreover, one long term growth experiment over 72 hours was conducted to see the longer term changes in pH.

During these experiments a unique pH profile for both bacteria compared to each other and compared to the negative control could be found. At first the pH of both bacteria dropped from neutral to acidic which was followed by a rise in pH into alkaline levels at the end of the log phase. Further research is required to confirm these results with other bacteria and, more importantly, *in vivo*. More research objectives are given in 6. Future Directions.

The in this study investigated pH sensor offers great opportunities in chronic wound treatment. Once the pH profile can be approved and bacterial infections in chronic wounds are shown to follow a similar detectable pH profile as the one found in this study, the pH sensor technology can be integrated into wound dressings. It is possible to include the pH measurement in a multi-parameter sensor included in wound dressings to continuously measure wound parameters. This way further trauma to the wound due to dressing removal can be reduced which saves patient from unnecessary pain and discomfort, bacterial infections can be identifies and treated before they become critical and delay the wound healing.

It is also imaginable that this technology can be adapted for critical bacterial count detection in burn wounds or even normally healing wounds. This would widen the scope of the application possibilities even further incentivising continued research.

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